# *MODES OF DRUG ACTION*

A GENERAL DISCUSSION

HELD BY

The Faraday Society

September, 1943



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1. drugs (2). malaria parasites

3. narcotics.

4. chemotherapeutic agents

5. insecticidal action

6. trypanosomes

7. ~~benzp~~ benzpyrene

8. lactis aerogenes

9. sulphonamides.

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# MODES OF DRUG ACTION

## A GENERAL DISCUSSION

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# MODES OF DRUG ACTION.

## A GENERAL DISCUSSION.

*Friday, 24th September, 1943.*

A General Discussion of the Faraday Society was held at The Hotel Rembrandt, London, S.W. 7, on Friday, 24th September, 1943, from 10 a.m. to 5 p.m. to discuss "Modes of Drug Action".

The President, Professor E. K. Rideal, occupied the Chair throughout the meeting. Some 250 members and guests of the Society were present. Luncheon was taken in the Hotel during an interval in the meeting. At the conclusion of the meeting thanks were accorded with acclamation to the Imperial College of Science for providing the epidiascope and other conveniences for the meeting at the hotel, as well as to the authors of the papers which had stimulated so useful a discussion.

The papers submitted in Advance Proof for discussion and the report of the discussion thereon appear in the following pages.

## GENERAL INTRODUCTORY ADDRESS.

By Sir Henry Dale.

The Faraday Society have honoured me with an invitation to open the proceedings at this day's Conference, and I have gladly accepted it; and I have done so the more gladly on account of the association of to-day's proceedings with the memory of my own old teacher and greatly honoured friend, the late Sir William Hardy. I am glad also to have the opportunity of adding my voice to those of other research workers in medicine and pharmacology, in welcome of the growing interest shown by physical chemists in many of our central problems. As evidence of this, we find the Faraday Society giving a day to the discussion of the actions of drugs, and we have the leaders of some of our chief schools of physical chemistry, with their co-workers, contributing to what promises to be a varied and illuminating discussion. It is almost a commonplace to say that any account of the life process, in its various manifestations, which science may eventually be able to present, will be written in terms of extremely complicated and labile physico-chemical systems. When we study the effects of chemical substances on different vital activities, then, and try to understand how these are modified, suspended or abolished by what we call pharmacological actions, we shall certainly need all that physical chemistry can contribute to our effort to understand what is happening. We are glad, accordingly, that this discussion is being held, and grateful to the Faraday Society.

Having said this, I hope that it will not seem grudging or presumptuous if I venture to suggest that those of us who have hitherto approached these problems, whether from the side of biology or of chemistry—and certainly myself no less than others, when I have rashly waded up to the short limit of my depth in these difficult waters—have tended almost inevitably to oversimplify the issue. I suspect that we have tried to attribute to such parallels between physico-chemical properties and pharmacological action, as we can observe in homologous series, a significance beyond what they can properly bear. Significance, of course, these parallels have; since any kind of vital process is dependent on

change in complex physico-chemical systems, the *intensity* of a particular action upon it, observed in an homologous series, is likely to be conditioned by some physico-chemical property that waxes and wanes again as we ascend the series ; and we seem, often enough, to have several such properties to choose from, with maxima at the same member of the series. The point which I wish to emphasise, however, is that this coincidence of maximum activity with a maximum of oil-solubility, or of activity on interfacial tension at a particular type of surface, may not throw any light at all, in the present state of our knowledge, on the specific appearance in that series of the physiological activity which we are studying, and which may be a highly specialised and an extremely complicated one. A good example of my meaning recurs in the papers to be presented by Dr. Schulman and Dr. Ing, both of whom happen to cite the interesting maximum of oestrogenic activity at the di-ethyl substitution stage in the series of dihydroxystilbenes. Dr. Ing is interested in this maximum from the point of view of structural chemistry, and Dr. Schulman in its coincidence with the form most actively adsorbed on a protein interface. Now I am certainly not suggesting that this coincidence is not of interest and significance ; but I do venture to urge that we should not allow ourselves, even subconsciously, to assume that it even begins to account for the occurrence, in this series as a whole, of the highly specific and complex activity, which we term oestrogenic. It is true that the formula of such a disubstituted stilbene can be written on paper in a shape recalling the carbon skeleton of the natural oestrogenic hormones ; but that, again, does not begin to tell us why the presence in the circulation of a trace of such a hormone, or of one of the members of the series under discussion, specifically initiates the complicated train of events in the mucous membrane of the female genital tracts which constitute what we call oestrus. That surely is not a general property of substances which are powerfully adsorbed on protein interfaces. All these parallels between structure, or physico-chemical properties, and special types of activity are of alluring interest and will, assuredly, provide data of great value for an eventual understanding of the problem, provided that we do not lose hold of the fact that its central core is still biological, and therefore of such complexity as still to be hardly tangible.

Paul Ehrlich, the real initiator and great pioneer of that side of to-day's subject which deals with the chemotherapy of infections, used to insist on the essentially biological nature of the problem, and to claim the initiative for the medical biologist. Yet the conceptions from which he sought to construct a framework of hypothesis, were largely derived from structural organic chemistry, and particularly from that of the dye-stuffs with which he was most familiar. And here again, it seems to me that the use of terms such as Ehrlich introduced, and others since his time, has its value as a scaffolding for ideas, provided that we remember that to label a phenomenon is not to reveal its nature. Dr. Ing alludes to the remarkable specificities of certain chemicals for physiologically effector cells—nerve, muscle and gland cells—and the association of these with innervation by different parts of the autonomic nervous system—surely one of the most fascinating of pharmacological mysteries, and one which remains a mystery, even with our present knowledge of the physiological intervention of adrenaline and acetylcholine in the transmission of different nervous effects. It is a mere statement of fact to say that the action of adrenaline picks out certain such effector-cells and leaves others unaffected ; it is a simple deduction that the affected cells have a special affinity of some kind for adrenaline ; but I doubt whether the attribution to such cells of " adrenaline-receptors " does more than re-state this deduction in another form.

Of great interest, again, is the switch-over as we ascend the " onium " series from predominant, selective stimulation to an equally selective paralysis of the same types of nerve cells and endings ; and this change

may legitimately be compared with a progressive change in various physico-chemical characters in the series. It seems to me, however, that, when we come to consider the basis of this selective action, we must not ignore anomalies, which, in the end, may prove to be more significant for any final conception than the regularities. The really fascinating problem, I suggest, is to be found in the fact that tetramethylammonium salts have a selective stimulant action closely similar to that of nicotine, of cytisine, and of lobeline, natural alkaloids which are not onium salts at all, and which are not remarkably similar to one another in molecular configuration. What is the property common to these, which gives them, in common, this highly selective activity ? To say that they all have affinity for the same chemoreceptors is merely to restate the observed facts or, if it means more, to go without warrant beyond them.

Dr. King justly draws attention, I think, to the interest of the facts obtained by the study of drug-resistant strains, selectively bred out, as it were, by dosage too low to effect a permanent cure of an infection. The mention of such matters brings to mind the great loss to the study of chemotherapy in this country entailed by the recent death of our distinguished colleague and brilliant leader in this field of enquiry, the late Prof. Warrington Yorke. Knowing that he was a doomed man, Yorke used himself to the very limit of his waning strength to serve the cause of our country and its allies. Prof. Yorke, and others who worked with him, or have since followed the trail, have been able to show that trypanosomes thus made resistant to a group of arsenicals have lost the affinity for them, which enables normal trypanosomes to extract these compounds from a surrounding fluid. The fact that other organic arsenicals are still fully active on these resistant trypanosomes, is interpreted by the presence in these compounds of lipoid-soluble groups. The trypanosomes which have thus been made resistant to atoxyl have also, as Ehrlich and his co-workers discovered, become resistant to acridine dyes like acriflavine, which no longer stain them as they do normal trypansomes ; but when Dr. King interprets this event as meaning that atoxyl and its analogues, on the one hand, and the acridine dyes on the other, are " substantive for the same type of structure in the trypanosomes," I am inclined to ask whether this does more than state in other terms the observed and surprising fact. And here I should like to raise the question, whether the recently observed possibility, of producing from normally sensitive cocci strains which have acquired a resistance to sulphonamide derivatives, may not present the opportunity for a direct attempt to discover something more definite about the chemical basis of such resistance. Unlike trypanosomes, such bacteria might, I suggest, be grown on a scale sufficiently large to enable some quantitative data to be obtained concerning certain constituents of the resistant and normally sensitive strains.

I hope that my remarks will not appear to be wholly critical and discouraging ; that is certainly not my feeling or my intention. On the contrary, I feel that the present position of the subject of this Conference represents a tremendous advance on that of only a few years ago. We shall have abundant evidence in this discussion of the different kinds of technique and the different orders of conception which are now being brought to bear, in a convergent attack on a problem to which, even quite recently, the only method of approach seemed to be that of hit or miss, an empirical and almost undiscriminating trial of any accessible derivative of a substance in which, whether by planned investigation or sheer accident, a particular type of activity had been discovered. But now, in place of vaguely conceived chemoreceptors, labels for observed but unexplained affinities, it begins to be possible to think in terms of interference with activities of vital enzyme systems, whether by blocking the action of co-enzymes, or supplanting essential substrate molecules on the specific surfaces of enzymes. We shall hear from Sir Rickard Christophers about the respiratory enzymes of malarial parasites and the effects on them of

antimalarial agents ; and there is the recent evidence about the mode of action of the sulphonamides and other bacteriostatic agents, as described by Fildes, Woods, MacIlwain and others, and discussed also to-day by Dr. King, in relation to the action of arsenoxides.

Then there are special problems, like those of the insecticidal actions discussed by Dr. Hurst, who has the advantage that he can skin his parasites and examine the separated cuticle for its properties as a physico-chemical system ; or that of the special properties, discussed by Dr. Lourie, which enable a trypanocidal agent to pass the so-called blood-brain barrier and become effective on trypanosomes infecting the brain. These may serve to illustrate the almost infinite complexities of the problem, viewed as a whole, on the biological side, and the need, if I may relapse into warning, for care not to assume too general a significance for the results obtained with a particular biological index, or an artificially simplified physico-chemical system or model. The present convergent attack, from so many special aspects of knowledge, gives, I believe, the highest promise of rapid and ordered advance in a field in which even opportunist and guerrilla tactics have already, and surprisingly, achieved results of such great practical importance.

# INTRODUCTORY ADDRESS.

## PART I. BIOLOGICAL ASPECTS: THE ANTAGONISM OF DRUGS.

By J. H. Gaddum.

*Communicated on 24th September, 1943.*

I should like to start by paying a tribute to Professor A. J. Clark. The title of this discussion is almost identical with that of a book which he wrote, and which, together with his article on General Pharmacology in *Heffter's Handbuch* [1] has been a great source of inspiration and information to all who are interested in pharmacology: I shall refer to them frequently.

The word drug means different things to different people. Many only use this word when they are speaking of dangerous drugs, such as opium, which lead to addiction. The pharmacologist likes to think that it means any substance which is applied to living matter, in order to see what effect it will have, and of course that means that there are very few substances which have not qualified as drugs at one time or another.

There are many modes of drug action. Some drugs act osmotically, some as acids or alkalis, or oxidising or reducing agents or by increasing the permeability of surfaces or precipitating proteins; some drugs act specifically on enzymes and some by quite unknown methods. We shall have a chance to discuss some complex modes of action this afternoon.

Many of the more interesting properties of drugs depend on the factors which cause them to concentrate at their site of action and several papers to-day are concerned with this process. Dr. Lourie is interested in the uptake of drugs in the central nervous system, Dr. King with their uptake by trypanosomes, and Dr. Hurst with their uptake by insects.

The actions of narcotics were discussed by this Society in 1937.[2] They are mostly simple aliphatic compounds which cause a reversible depression of living tissues. Their action depends on their general physical properties which cause them to concentrate on surfaces and in lipoids. The exact reasons for this concentration have been much discussed, but comparatively little is known about how these drugs depress living tissues once they have accumulated on their surfaces.

According to Dr. King [3] phenylarsenoxide is taken up by trypanosomes in much the same way as narcotics are taken up. The uptake of atoxyl, and other drugs to which trypanosomes become resistant, depends on more specific chemical properties, and the same is true of many of the more interesting drugs, which combine specifically with tissues. Dr. King's other class of arsenical drugs are those which are comparatively inactive and are perhaps not concentrated at all.

I do not propose to review all possible modes of drug action, but to concentrate attention on the antagonism of drugs with reversible actions, since many recent developments are based on the quantitative study of antagonism, and there is undoubtedly much more work to be done in this field.

An antidote may counteract the effects of a poison in various different ways, the two most important of which are by neutralisation and by competition. It may either neutralise the poison by combining with it and forming an inert compound, or it may compete with the poison for chemical groups which are in some way essential to living tissue.

[1] Clark, *Heffter's Handbuch. exp. Pharmak. Ergänz.* 4 (Springer, Berlin, 1937).
[2] *Trans. Faraday Soc.*, 1937, 1057. [3] *Vide infra*, 383.

## Antagonism by Neutralisation.

An example of an antidote which acts by neutralisation is provided by compounds containing —SH groups, which counteract the effects of Hg and other heavy metals, and of organic arsenic compounds. It was shown in 1908 by Chick [4] and has repeatedly been confirmed [1] that the action of mercury on bacteria can be reversed by treating the bacteria with $H_2S$. The initial reaction between the bacterium and the mercury is evidently reversible, though irreversible changes take place later. In the case of spores the reversible phase may last a week.

Soon after the last war Voeghtlin [5] and various collaborators were studying the actions of aromatic arsenic compounds in the chemotherapy of trypanosome infections. They showed that the action of trivalent arsenoxides of the form R – As = O could be directly observed by watching the movements of the trypanosomes under a microscope. When the arsenical was added, even in low concentrations, the movements ceased. This crude method of observing the effects of drugs gave interesting information. New methods, such as those described by Sir Rickard Christophers [3] will doubtless give much more information.

The effect of trivalent arsenicals on the movements of trypanosomes was antagonised by adding any one of a number of SH— compounds. The same effect was demonstrated *in vivo*, by injecting SH— compounds into infected rats 1 minute before the injection of arsenoxides. This caused a delay in the disappearance of trypanosomes from the blood. It was also shown that SH— compounds prolonged the life of rats which had received a lethal dose of arsenoxide. This work has been confirmed and extended.[1, 6] Voeghtlin believed that the arsenoxides were working by combining with the SH— groups which could be detected in the trypanosomes by the nitroprusside test. There can be little doubt that this combination takes place, since SH— groups have a particularly high affinity for arsenicals, but when this theory was advanced there was no direct evidence that the loss of free SH— groups would harm the organisms.

Fildes [7] has shown that the action of Hg on Bact. coli is also antagonised by various SH— compounds, and has suggested that Hg also acts by combining with SH— groups in the organisms. The affinity of SH— compounds for Hg is of course great and has earned for them the title of mercaptans, but Fildes points out that the evidence is now fortified by work on the nutrition of bacteria. Dr. Fildes has taken a leading part in this work himself and it is unfortunate he is unable to give an account of it to-day (*cf.* Fildes [8]). Bacterium coli can grow when the only source of nitrogen in the medium is ammonia, but B. typhosum cannot grow, except when certain amino acids are added. Both organisms contain these amino acids as an essential part of their structure, but one can synthesise them from ammonia and the other cannot. In Fildes's terminology these amino acids are "essential metabolites" for both organisms; they are "growth factors" for B. typhosum, which cannot synthesise them, but not for B. coli which can. These investigations have thrown much light on bacterial metabolism, since some organisms will only grow when supplied with very elaborate growth factors.

It has been shown that moulds [9] and staphylococcus [10] and presumably other cells as well can obtain sulphur from any one of a variety of organic SH— compounds, presumably by converting them into cysteine, but cannot grow at all without SH— compounds. Further references to

[4] Chick, *J. Hyg.*, 1908, **8**, 92.
[5] Voeghtlin, *Physiol. Rev.*, 1925, **5**, 63.
[6] Walker, *Biochem. J.*, 1928, **22**, 292.
[7] Fildes, *Brit. J. exp. Path.*, 1940, **21**, 67.
[8] Fildes, *Lancet*, 1940, **1**, 955.
[9] Volkonsky, *Ann. Inst. Pasteur*, 1934, **52**, 76.
[10] Fildes and Richardson, *Brit. J. exp. Path.*, 1937, **18**, 292.

chemical evidence of the importance of SH— compounds have been given by King.[3] There is thus good evidence not only that Hg is likely to combine with SH— compounds, but that this combination will inhibit growth. According to Fildes removal of SH— compounds is the essential cause of the inhibition of growth by Hg.

## Quantitative Theory of Neutralisation.

The quantitative relations between the concentrations of the poison and its antidote which just neutralise one another cannot be very accurately measured, but they can be studied over a wide range of concentrations. Voeghtlin found that the concentration of antidote had to be 10-20 times as great as the chemical equivalent of the poison. Fildes found that the corresponding ratio was 1·5-2 for glutathione and 6-12 for thiolacetate, and points out that this difference cannot be due to a difference of stability, since thiolacetate is more stable than glutathione under the conditions of the experiment. It seems likely that the difference is due to a difference in the dissociation constants of the compounds formed by combination of Hg with the two different antidotes.

Calculations may be based on two different fundamental assumptions. In the first place it may be assumed that it is necessary to use enough antidote to reduce the concentration of free poison to the level which would just cause a threshold effect in the absence of antidote. This is not Fildes's theory, since he classifies all SH— groups together as an essential metabolite whose absence would itself inhibit growth. The second assumption is therefore that it is necessary to use enough antidote to maintain a threshold concentration of free antidote.

Let $m$ molecules of the poison $X$ combine with $n$ molecules of the antidote $Y$ to form an inactive compound $XY$, and let $x$ and $y$ be the total molar concentrations of $X$ and $Y$. Consider the threshold conditions in which there is just enough free $X$ to have an effect. Let the concentration of $X$ which is not combined with $Y$ in these conditions be $x_0$, of which $x_1$ is free and $(x_0 - x_1)$ is combined with the tissue. On the first assumption these quantities are independent of $x$ and $y$. The concentration of the inactive compound will be $\frac{(x - x_0)}{m}$ molar, and the concentration of free $Y$ will therefore be

$$y - \frac{n}{m}(x - x_0).$$

For equilibrium

$$x_1{}^m\left[y - \frac{n}{m}(x - x_0)\right]^n = K\frac{(x - x_0)}{m}$$

where $K$ is the dissociation constant of $XY$.

$$\therefore\ y = \frac{n}{m}(x - x_0) + \left[\frac{K}{x_1{}^m} \cdot \frac{x - x_0}{m}\right]^{\frac{1}{n}} \quad . \quad . \quad . \quad (1)$$

The relation may be expressed graphically by plotting $y$ against $x$ (see Fig. 1). The line AE, which expresses conditions giving equal effects is called an isobol.[11, 12] The straight line AB represents the case where dissociation is negligible; $K = 0$ and $y = \frac{n}{m}(x - x_0)$. Under these conditions $Y$ is all combined with $X$ and the uncombined $X$ is merely the excess. The last term in equation (1) is represented by ED. This is the extra amount of antidote which must be added to suppress the tendency to dissociation.

[11] Fraser, *Brit. Med. J.*, 1872, **ii**, 457, 485.
[12] Loewe, *Ergeb. Physiol.*, 1928, **27**, 47.

When $n = 1$, that is when there is only one molecule of antidote in each molecule of the inert compound, AE is a straight line, and the concentration of antidote is directly proportional to the concentration of poison, provided that the latter is corrected by subtracting the amount required in the absence of antidote. When $n = 2$ the curve connecting ED and $x$ is a parabola, and as $n$ becomes longer the relationship diverges more from that of simple proportionality. These statements are true whatever the value of $m$, which has no effect because $x_1$ is assumed constant.

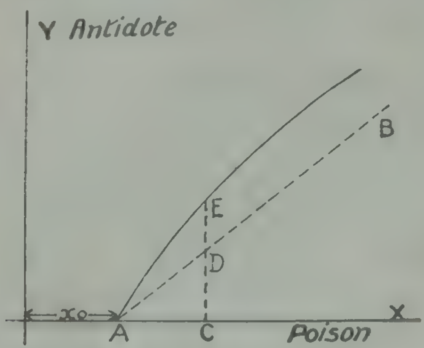


FIG. 1.—AE is the isobol, showing concentrations of poison and a neutralising antidote which have a constant effect. Arithmetic scales.

According to this theory the amount of antidote always exceeds the simple chemical equivalent of the amount of poison, provided that the latter is corrected as before. This is in accordance with known facts. If $n > 1$ the ratio $\frac{x - x_0}{y}$ decreases as the concentrations increase. The equation for the theory that the effect depends on the removal of excess antidote can be derived from equation (1) by interchanging $x$ with $y$ and $m$ with $n$. According to this theory the amount of poison always exceeds the simple chemical equivalent of amount of antidote, corrected as before.

The observations of Voeghtlin and Fildes that a large excess of antidote was sometimes necessary suggest that the second assumption is incorrect in these cases, and that the function of the added SH— compounds is to remove the poison rather than to maintain a threshold concentration of free SH— groups. On the other hand Woods [13] found that a substance in yeast antagonised sulphanilamide in remarkably low concentrations, and was led to the conclusion that the antagonism was due to competition. There is no doubt that this conclusion was correct, but the above discussion suggests that it was illogical. If $K$ was large, even a small amount of antidote might be sufficient to maintain a threshold concentration of free antidote. A large excess of antidote will only be needed if it is necessary to keep down the concentration of free poison.

This method of calculation is only useful when the total amounts of poison and antidote are known and the amounts free cannot be independently estimated. When this can be done it should be possible to show by direct measurement that the concentration of free poison, or of free antidote, remains constant.

Another important class of antidotes which act by combining with poisons includes the antibodies which appear in the serum of immunised animals. The quantitative aspects of this combination have been discussed by Marrack.[14] There are, of course, other examples.

The general conclusion is that the study of neutralising antidotes is unlikely to lead to new pharmacological knowledge, since they only act by reducing the concentration of free poison, and give no information about the action of the poison different from that obtained by reducing the concentration in other ways.

[13] Woods, *Brit. J. Exp. Path.*, 1940, **21**, 74.
[14] Marrack, 1939. *The Chemistry of Antigens and Antibodies*, M.R.C. Spec. Rep. Ser. No. 230.

## Antagonism by Competition.

It is more profitable and interesting to study the drugs which antagonise one another by competition, since it is possible in this way to find out something about the groups for which they compete. A study of competitive antagonisms also leads to a convenient classification of drugs, since those drugs which have the same antagonists probably act on the same receptors. The word receptors is used here to denote the site of action of the drug and does not imply any theory of its nature.

When a drug has combined with the appropriate receptors in a tissue it may either produce a pharmacological response or it may do nothing but block up the receptors and so exclude active drugs.

This process of competition has been attracting attention in many ways. It has long been known to students of enzymes.[15] Hydrogen ions may compete with enzyme poisons, such heavy metals [16] or basic dyes,[17] and hydroxyl ions may compete with acid dyes. Substances allied to the normal substrates of enzymes may also cause competitive inhibition. The pharmacological importance of competition was emphasised by evidence that the actions of eserine were due to the accumulation of acetylcholine preserved by competition from destruction by cholinesterase [18] and that the actions of ephedrine were similarly due to the preservation of adrenaline.[19] Much work has been done on the antagonism between pairs of drugs, such as atropine and acetylcholine, or ergotoxine and adrenaline, which compete for the pharmacological receptors near nerve endings.[1]

The evidence obtained by Woods,[13] working in Fildes's laboratory, that the action of sulphanilamide is due to competition between the drug and *p*-amino-benzoic acid has attracted widespread attention and been generally accepted. It led to the discovery that *p*-aminobenzoic acid is a growth factor for certain micro-organisms,[20] and also mammals : it has, for example, been found to prevent grey hair in rats fed on a deficient diet,[21] and in cats poisoned with hydroquinone.[22] Dr. McIlwain has given us an able review of the developments in this field, including his own striking results, obtained after Dr. Fildes, and other members of the team, became engaged on work in connection with the war.

## The Combination of Drug and Tissue.

Dr. Ing [3] has given us an interesting account of the ways in which molecules have been stripped of unnecessary coverings revealing the essential skeleton of what he calls pharmacodynamic groups. These groups are sometimes present both in poisons and in their antidotes and are therefore probably important for the combination of the drug with the appropriate receptors, rather than for its subsequent effect. It is possible that they represent the part of the drug which combines with the receptors by means of true valencies.

Many drugs are bases and there is some evidence that tissues can remove such drugs from solutions by a process of base-exchange such as occurs with permutit.[23] In some cases there are two or more groups which probably combine by true valencies and the distance of these apart may

[15] Haldane, *Enzymes* (Longmans Green, 1930).
[16] Myrbäck, *Z. physiol. Chem.*, 1926, **158**, 160.
[17] Quastel and Yates, *Enzymologia*, 1936, **1**, 60.
[18] Stedman and Stedman, *Biochem. J.*, 1931, **25**, 1147.
[19] Gaddum and Kwiatkowski, *J. Physiol.*, 1938-39, **94**, 87.
[20] Rubbo and Gillespie, *Nature*, 1940, **146**, 838.
[21] Ansbacher, *Science*, 1941, **93**, 164.
[22] Martin and Ansbacher, *J. Biol. Chem.*, 1941, **138**, 441.
[23] Zipf, *Arch. exp. Path. Pharmak.*, 1927, **124**, 259.

be critical. The very specific effects of low concentrations of some drugs must depend on a very elaborate fit of the key in the lock, which I suppose depends on polar effects and on the shape of the rest of the molecule. When the fit is good enough, the receptors become saturated in the presence of particularly low concentrations of the drug which is then said to be very active.

On the other hand I do not agree with the view that the activity of a drug probably depends on the ease with which it can combine with receptors. The competition which has been most accurately studied is the competition between oxygen and carbon monoxide for hæmoglobin. Both gases combine with hæmoglobin in exactly the same way and their dissociation curves are identical, except that the dissociation constant for $O_2$ is about 240 times that for CO. This constant represents a balance of the opposing processes by which the gases combine with hæmoglobin and dissociate again. Roughton [24] has shown that both combination and dissociation are slower for CO than for $O_2$, but that the dominating factor is the high stability of carboxyhæmoglobin. Owing to this stability, CO is about 240 times as active a drug as $O_2$. It seems to me likely that most highly active and specific drugs are also active because they form stable compounds with the tissues. An elaborate fitting together of molecules is clearly necessary and this is more likely to lead to stable combination than to rapid combination.

Dr. Ing's views on optical isomers are interesting. If he is right it might be possible to demonstrate that perfused tissues remove the active isomer selectively from the perfusion fluid.

## Quantitative Theory of Competition.

A simple theory accounts for the quantitative facts of competition in many cases.[25] Let $C_1$ be the concentration of free active drug, or essential metabolite, or antidote, and let $C_2$ be the concentration of free poison competing for the receptors. Let $a$ and $b$ be the corresponding proportions of the receptors occupied by the two drugs, so that $(1 - a - b)$ is the proportion of the receptors free. Then for equilibrium

$$K_1C_1 (1 - a - b) = a \quad \text{and} \quad K_2C_2 (1 - a - b) = b.$$

Elimination of $b$ gives

$$K_1C_1 = (1 + K_2C_2) \frac{a}{1 - a}. \qquad (2)$$

When $C_2 = 0$ this reduces to a mass law equilibrium in its simplest form and has been used to express the uptake of oxygen by hæmoglobin, poisons or substrates by enzymes, and acetylcholine and adrenaline by pharmacological receptors. It is identical with the formula used by Langmuir [26] to express the adsorption of gases.[27] When plotted on an arithmetic scale of concentration it gives a hyperbola, and when plotted on a logarithmic scale it gives the symmetrical sigmoid curve, which is well known as the titration curve of a buffer. This logarithmic method of plotting is convenient because it shows the relation of the simplest curves to those that are less simple.

In the presence of a small amount of acid the curve connecting the concentration of oxygen and the percentage saturation becomes concave at low concentrations, but the curve obtained by plotting the logarithm of the concentration retains its symmetry and only becomes steeper than before. The results can be expressed with fair accuracy by replacing log $C$

[24] Roughton, *Proc. Roy. Soc. B.*, 1934, **115**, 451.
[25] Gaddum, *J. Physiol.*, 1937, **89**, 7P.
[26] Langmuir, *J. Amer. Chem. Soc.*, 1918, **40**, 1361.
[27] Hitchcock, *ibid.*, 1926, **48**, 2870.

by $n \log C$, or $C$ by $C^n$ where $n = 2{\cdot}5$ (Hill [28]). This suggests a reaction of the second or third order, and it was thought at one time that hæmoglobin must be in aggregates each of which could combine with $n$ molecules of oxygen. On this theory it is assumed that an aggregate cannot combine with a single molecule of oxygen, or if it does the compound so formed dissociates so rapidly as to make no significant contribution to the oxygen uptake. It was then shown, by observations of osmotic pressure and with the ultracentrifuge, that hæmoglobin dissolves in aggregates each of which could combine with 4 molecules of oxygen, so that $n$ should be 4. This was explained by Adair [29] on the theory that each aggregate can combine with anything up to 4 molecules of oxygen and that the stability of the combination increases as the number of molecules of oxygen in each aggregate increases. This theory resembles Hill's in suggesting that unsaturated aggregates are relatively unstable; it suggests that $n < 4$ because their stability is not negligible.

The application of formula (2) to the antagonism of drugs on the receptors at nerve endings has been studied in great detail.[1] The relation between the concentration ($C_1$) of adrenaline or acetylcholine and their effects on a wide range of tissues is well expressed by this formula, if it is assumed that the effect is proportional to $a$. When the effect is plotted against the logarithm of the concentration of antidote the curve is the same shape and slope, either in the presence, or in the absence, of antagonistic drugs. The effect of the latter is merely to move the curve horizontally without changing its shape. This is explained on the theory that the reaction between these active drugs and the receptors is always of the first order and that the pharmacological effect is directly proportional to the amount of drug combining with the receptors. On the other hand the combination between antagonists, such as ergotoxine and atropine, with the receptors is not so simple and the results can only be expressed by a formula of the form

$$K_1C_1 = [1 + (K_2C_2)^n] \frac{a}{1 - a}. \qquad (3)$$

In this formula $K_2$ represents the reciprocal of the concentration causing a doubling of $C_1$. The interaction of heavy metals and dyes and hydrogen ions are expressed by the same formulæ.[1]

The quantitative aspects of the antagonism of sulphanilamide and *p*-aminobenzoic acid were discussed by Wyss,[30] who arrived at an equation identical with equation (2) above, though with a different notation. He pointed out that the rate of growth should depend on $a$, and that his results indicated that the rate of growth in the exponential phase was directly proportional to $a$. According to Davis and Hinshelwood [3] the matter is more complicated than Wyss supposed, since there may be two exponential phases of growth in the presence of sulphanilamide, and growth never stops completely even in high concentrations of sulphanilamide.

The data given by Wyss for the effect of $C_2$ do not fit formula (2), but do fit formula (3) if $n = 2$. The results of Strauss *et al.*[31] appear to support this conclusion, since their results may be fitted by straight lines of slope 2, when $\log C_1$ is replotted against $\log C_2$. These data suggest that $C_1/C_2$ is not constant. On the other hand Wood [32] found that $C_1/C_2$ was constant and his data suggest that $n = 1$. Similar variations in $n$ occur in experiments with ergotoxine and adrenaline, or with atropine and acetylcholine.[1] They may perhaps be due to reactions of order different from 1, or to the fact that the concentration of the drugs at their

[28] Hill, *J. Physiol.*, 1910, **40**, 4P.
[29] Adair, *J. Biol. Chem.*, 1925, **63**, 529.
[30] Wyss, *Proc. Soc. exp. Biol.*, 1941, **48**, 122.
[31] Strauss, Lowell and Finland, *J. Clin. Invest.*, 1941, **20**, 189.
[32] Wood, *J. exp. Med.*, 1941, **75**, 369.

site of action is not directly proportional to their concentration in the liquids in contact with the tissue or to failure to correct for the threshold concentration.

## Comparison of Formulae for Neutralisation and Competition.

In comparing formula (3) with formula (1) it must be remembered that $C_1$ and $C_2$ represent concentrations of free drug, and $x$ and $y$ represent total concentrations. In experiments on competition it is sometimes possible, by maintaining a large excess of fluid in contact with the tissue, to ensure that the final free concentration is practically equal to the total concentration, but this is not always the case. Some of the variations in the value of $n$, discussed in the last paragraph, may be due to the assumption that $C_1$ and $C_2$ are equal to the total concentrations of the drugs. In any case it is clear that the relationships are similar in the two cases. When the reactions are of the first order the ratio of the concentrations is constant in either case, provided that a correction is made for the threshold concentration of one of the drugs. In neither case is there any theoretical limit to the value of this ratio. It would probably not be possible to distinguish between the two types of antagonism by studying the quantitative relationships, and this can only be determined from independent evidence that the drugs combine with one another, or would be likely to compete.

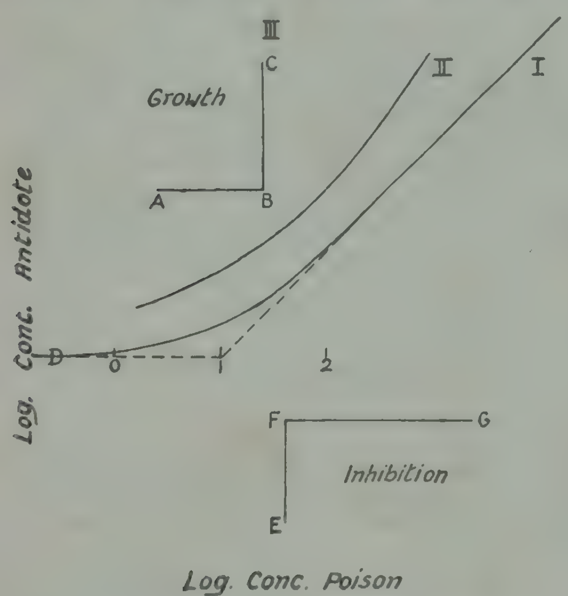


FIG. 2.—Different types of isobol separating conditions for growth (top left) from those for inhibition (bottom right). Logarithmic scales to base 10.

ABC. No evidence of specific antagonism.
D. Direct neutralisation or competition. Equations of first order. On arithmetic scales this would be linear.
EFG. Poison inhibits synthesis of an essential metabolite (the antidote).

## Other Types of Antagonism.

Fildes [33] has also drawn attention to other interesting types of relation between poison and antidote. Tryptophane is essential for the growth of bacteria, which normally obtain it by synthesis from indole. Indoleacrylate inhibits growth and the evidence suggests that it competes with a hypothetical substance intermediate in the synthetic chain between indole and tryptophane. This was shown by the fact that a small amount of tryptophane completely antagonised the inhibition, and the amount required was practically unaffected when the amount of indoleacrylate was increased 5 times (FG, Fig. 2). There was no evidence in these experiments that indole had any antagonistic action at all (ABC), but it might be expected that a more detailed analysis would reveal a certain amount of graded antagonism over a small range of low concentrations. Neither substance showed graded antagonism over a wide range of concentrations such as occurs with direct competition

[33] Fildes, *Brit. J. exp. Path.*, 1941, **22**, 293.

(curve D) and would presumably be given by the hypothetical intermediate substance.

In this type of diagram, graded antagonism is shown by a sloping isobol. With linear scales and reactions of the first order, the isobol is straight. Owing to the wide ranges of concentrations involved it is often necessary to use logarithmic scales. If the reaction is of the first order the isobol is then as shown in curve D (Fig. 2) with a slope of 45°. If the isobol becomes parallel to one of the axes at high concentrations (BC or FG) it suggests that the concentration of the corresponding drug has risen above the threshold value required to produce a maximal effect, such as saturation of an enzyme. If the curve becomes parallel to one of the axes at low concentrations (AB or EF) it suggests that the opposite drug has fallen below a threshold value. If the actions of the two drugs are completely independent a curve such as ABC is expected.

Other examples of antagonism have been studied by McIlwain [34] who classifies competition as type I of antagonism, and results like those with indole as type III, and describes an intermediate type II, but says nothing about the type of antagonism shown by tryptophane and indoleacrylic acid.

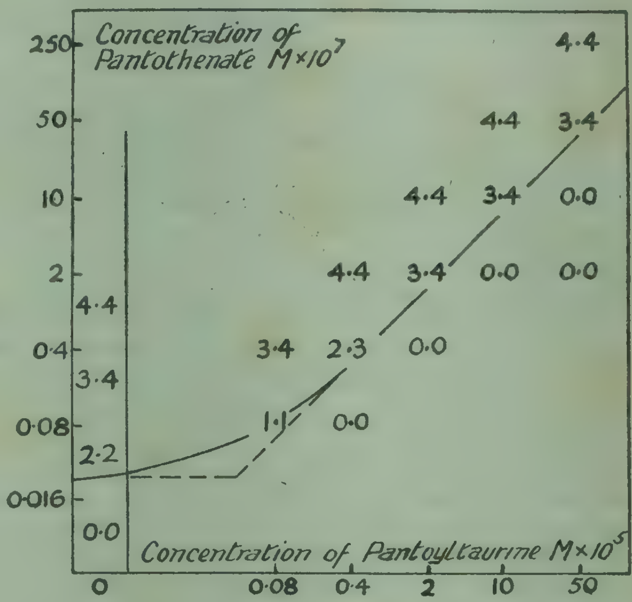


FIG. 3.—A method of tabulating results to show their quantitative relations. The figures denote the growth observed at two different times. Isobols are drawn between the figures to represent any given amount of growth. In this case the antagonism is due to competition (McIlwain's data).

The competition of $O_2$ and CO for Hb shows a curious paradox, since at low concentrations in the presence of acid the addition of one gas may increase the combination of the other with Hb.[35] In this region, where the dissociation curve is concave upwards, doubling the concentration of either gas, taken alone more than doubles the percentage saturation of the hæmoglobin. If, therefore, the two gases are present together in equivalent concentrations the percentage saturation will also be more than doubled, and, since equal quantities of each combines, each increases the uptake of the other.

## Conclusion.

The high activity and specificity of the action of many drugs depend on the intermolecular forces which bind them to appropriate receptors. As with the narcotics, we know comparatively little about how they really act once they have been concentrated at the site of action. In many

[34] McIlwain, *Brit. J. exp. Path.*, 1940, **21**, 136; 1941, **22**, 148; 1942, **23**, 95.
[35] Barcroft, *Respiratory Functions of the Blood* (Cambridge University Press, 1928).

cases this is a problem for students of enzymes, and it is to be hoped that they will continue to collaborate with pharmacologists in this field. This collaboration has widened our knowledge both of drugs and of enzymes.

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## *GENERAL DISCUSSION.*

**Dr. H. McIlwain** (*Sheffield*) (*communicated*): I agree with Professor Gaddum that the study of drug-antagonism has proved an extremely valuable means of analysing the effects of drugs. I would like to emphasise an aspect of the subject which is complementary to that discussed by him, and which perhaps alters the balance of his conclusion (on p. 327) that the antagonism of drugs by neutralisation is a less profitable or interesting study than their antagonism by competition. The aspect in question is the natural occurrence of antagonistic substances. Thus, the finding that a particular type of compound is the most potent antagonist which can be extracted from the biological system affected, or from other natural sources, has an important bearing on the mode of action of the drug whether that be by neutralisation or by competition.[36] In both cases the compound isolated has dual significance: its relation to the drug and to natural processes taking place independently of the drug. It is then a useful working hypothesis to assume that the processes affected by the drug are related to ones in which the antagonist, isolated from natural sources, takes part. Such studies have suggested acriflavine to act by combination with nucleotides,[37] and mercuric salts by combination with sulphur-containing proteins.[38] Without such analysis of natural systems, the selection of compounds for testing as antagonists is arbitrary, and those acting by neutralising may well have little significance. This was illustrated [36] by considering the systems which might be affected by hydrochloric acid, whose antagonism by sodium hydroxide has little biological significance, while its antagonism by natural buffers and basic groups, has such significance.

In investigating natural materials as drug-antagonists one must consider a wider array of modes of interaction than those selected by Professor Gaddum or enumerated in an earlier paper.[39] Four modes examined recently [40] in such a study were the following: (1) Reactions between inhibitor and antagonist in the absence of the biological system. (2) Reactions in the presence of the biological system. These two may include the combination referred to by Professor Gaddum, but can occur through types of reaction between inhibitor and antagonist, other than their combination; for example, inactivation of an inhibitor occurred through its reduction in the presence of the biological system together with antagonists, which probably functioned as hydrogen carriers. (3) Neutralisation of effects, but not necessarily chemical reaction between the two components, through independent actions on the biological system; for example, the antagonism of substances inhibiting bacterial growth, by others which independently promote it. (4) Neutralisation, but not chemical reaction between the two components, through related actions on the biological system. This would include such examples as the antagonism by methionine of the antibacterial action of sulphanilamide, as well as its antagonism

[36] McIlwain, *Lancet*, 1942, **1**, 412.
[37] McIlwain, *Biochem. J.*, 1941, **35**, 1311.
[38] Smith, Czarnetzky and Mudd, *Amer. J. Med. Sci.*, 1936, **192**, 790.
[39] McIlwain, *Brit. J. exp. Path.*, 1940, **21**, 136.
[40] McIlwain, *Biochem. J.*, 1943, **37**, 265.

by *p*-amino-benzoate. The four modes of interaction are placed in order of increasing specificity of interaction with the biological system and clearly admit of intermediate cases.

I find very valuable Professor Gaddum's tabular representation of the interaction of drug and antagonist (*cf.* [40]), and am certainly in agreement in recognising the distinctive character of the interaction of indoleacrylate with tryptophan. This (then unpublished) was not included with other types enumerated in 1940,[39] as that analysis was of specific experimental results, but an analogous interaction is discussed later,[42] and discriminated from other cases. The types [39] were characterised by certain quantitative relations, and overlap with the above classification in terms of relationship to the biological component.

[41] Kohn and Harris, *J. Pharm. exp. Ther.*, 1943, **77**, 1.
[42] McIlwain, *Biochem. J.*, 1942, 417.

# THE CELL METABOLISM OF THE MALARIA PARASITE IN RELATION TO THE MODE OF ACTION OF ANTIMALARIAL DRUGS.

By S. R. Christophers.

*Received 9th August*, 1943.

In our investigation of the mode of action of antimalarial drugs it seemed probable to Dr. Fulton and myself that antimalarials of the quinine type might function through the inhibition of the enzymes responsible for the cell metabolism of the parasite. We therefore took up the study of this question. We were in a good position to do so since we had been dealing for some time with parasite material as obtained from *Macacus rhesus* monkeys heavily infected with *Plasmodium knowlesi.*

Since we obtained interesting and suggestive results,[1, 2] and others, especially Coggeshall and Maier,[3, 4, 5] Velick [6] and more recently Wendel [7] have confirmed and extended these results, a brief summary of and comment on this line of work seems desirable.

The material mainly employed has been that obtained from *Macacus rhesus* heavily infected with *P. knowlesi.* But other monkey parasites, *P. inui* and *P. cynomolgi*, as also the bird parasites, *P. cathemerium* and *P. lophurae* have been studied in this connection by some authors. Such material may be either the centrifugally separated and washed large forms of the parasite suspended in a suitable medium, or, as originally pointed out by us,[1] whole blood will give satisfactory readings. When whole blood, defibrinated or heparinised, is used, all the elements of the blood including non-parasitised cells are present and the readings show difference between infected and normal blood. In the separated parasites obtained by centrifuging, remnants of the red cell surrounding the parasites are still present amounting usually to about one quarter of the material. Parasites completely free from red cell and apparently but little damaged can, however, be obtained, as shown by Fulton and myself,[8] through

[1] Christophers and Fulton, *Ann. Trop. Med. and Par.*, 1938, **32**, 43.
[2] Fulton and Christophers, *ibid.*, 77.
[3] Coggeshall, *J. Exper. Med.*, 1940, **71**, 13.
[4] Coggeshall and Maier, *J. Inf. Dis.*, 1941, **69**, 108.
[5] Maier and Coggeshall, *ibid.*, 87.
[6] Velick, *Amer. J. Hyg.*, 1942, **35**, 152.
[7] Wendel, *J. Biol. Chem.*, 1943, **148**, 21.
[8] Christophers and Fulton, *Ann. Trop. Med. and Par.*, 1939, **33**, 161.

saponisation of the deposit. In all these forms of material $O_2$ uptake can be demonstrated and measured.

The method used for determining $O_2$ uptake has been by the Barcroft differential manometer, usually under aerobic conditions at 37° C.

It has been established up to date that

(1) Suspensions of parasitised cell deposit, or heavily infected whole blood, consumes $O_2$ at a rate very much greater than that which could arise from either the red cell substance or plasma present. Wendel [7] notes that parasitised cells in one specimen consumed $O_2$ about 300 times as rapidly during the first measured period as do cells in normal blood.

(2) Uptake occurs with only a moderate reduction using washed saponised material containing no other elements than the parasites, provided glucose be present.

(3) Uptake is proportional to the amount of parasite deposit or the number of parasites in a given stage of development.

(4) Uptake is much greater with the larger forms of the parasite than with the young stage. Velick [6] working with *P. cathemerium* found uptake with heavily infected whole blood up to ten times greater in the later stages of the developmental cycle than at its commencement when the parasites were small.

(5) Uptake can be inhibited by even minute traces of certain drugs.

There would seem to be no reasonable doubt but that the very considerable $O_2$ uptake is due to respiratory metabolism of the parasite.

Changes associated with respiration by trypanosomes have been investigated by a number of observers following upon the pioneer observations on glucose and oxygen consumption by Nauss and Yorke.[9] $O_2$ uptake by these organisms has been investigated by Fenyvessy and Reiner,[10, 11] Kudicke and Evers,[12] Reiner, Smythe and Pedlow,[13] and Christophers and Fulton.[1] It will be sufficient for our purpose to give the main facts that have been established. A suspension of trypanosomes put up under similar conditions to those employed for the malaria parasites shows an enormous $O_2$ uptake. With a strain of *T. rhodesiense* Dr. Fulton and myself found an $O_2$ uptake during the first half hour at the rate of 19·4 ml. per hour for $10^{10}$ trypanosomes or determined on dried weight $Qo_2 = 285$. If no glucose is added to the suspension this rate rapidly falls off and in quarter to half an hour from the using up of such glucose as may have been present in the serum, *i.e.*, in about half an hour's time, all uptake ceases. Even if a considerable amount of glucose be added, say 0·2 per cent., uptake after an hour or so falls and rapidly comes to an end as this amount is exhausted. During this process in which glucose is used up acid is formed. We found just under 1 molecule of glucose (0·9) used up and 2 molecules of monobasic acid formed for each molecule of $O_2$ taken up. $CO_2$ is formed but in small amount, the respiratory quotient for *T. rhodesiense* found by us being 0·155. These results are closely parallel to those previously given by Reiner, Smythe and Pedlow [12] for *T. equiperdum*. These authors found the chief products obtained to be glycerol anærobically and pyruvic acid ærobically. The respiratory quotient was again small, *viz.*, 0·062, though for *T. lewisi*, a different type of trypanosome, it was approximately 1·0.

Coming to observations on the malaria parasite $O_2$ uptake is likewise very considerable. Dr. Fulton and myself found it to be in the first half hour at the rate of 2·17 ml. per $10^{10}$ parasites per hour, or 8-9 times smaller than that for an equal number of trypanosomes, which, however, parasite for parasite would be considerably larger. $Qo_2$ based on dried substance

[9] Nauss and Yorke, ***Ann. Trop. Med. and Par.***, 1911, **5**, 199.
[10] Fenyvessy and Reiner, *Z. Hyg.*, 1924, **102**, 109.
[11] Fenyvessy and Reiner, *Biochem. Z.*, 1928, **202**, 75.
[12] Kudicke and Evers, *Z. Hyg.*, 1924, **101**, 317.
[13] Reiner, Smythe and Pedlow, *J. Biol. Chem.*, 1936, **113**, 75.

as determined after saponisation was 35, again about 8 times smaller than that for trypanosomes. Nevertheless, though considerably smaller than that for trypanosomes, $O_2$ uptake for malaria parasite substance is relatively quite large as compared with figures given by different observers for tissue determinations. Our figure of $Qo_2 = 35$ was the mean of a large number of experiments using full grown or nearly full grown parasites. Wendel [7] working with parasites in different stages of development found $O_2$ consumption in 35 samples to vary between 157 and 2280 ml. per $10^{14}$ cells per hour, uptake being greatest with the large presegmenter forms. Velick [6] using whole blood with *P. cathemerium* found at the stage of maximum development of the parasite cycle a difference up to 10 times the uptake when the parasites were small.

A curious feature of $O_2$ uptake by parasites, and in marked contrast to that by trypanosomes, is that uptake continues apparently indefinitely whether extra glucose has been added or not. There is never the rapid fall off due to exhaustion of the glucose seen in trypanosome material. Indeed this steady uptake continued for many hours (as much in our experience as 10 hours on one occasion) is a very characteristic feature of $O_2$ uptake by parasites. Nevertheless glucose when present is utilised.

That the continued uptake is merely due to there being sufficient glucose normally present in the serum or red cells does not seem to be the explanation. Wendel says " glucose initially present in a sample of moderately or highly parasitised blood is destroyed during the first hour of incubation *in vitro*, yet at such time *P. knowlesi* may continue respiring at a constant or even accelerated rate." It is not due to glycolysis by normal blood, as this author found, as we did, that the blood of normal monkeys and normal red cells from infected monkeys destroys glucose quite slowly. According to Wendel $10^{14}$ normal cells destroys glucose at an average rate of 20 mg. per hour at 37° C., whereas parasitised cells destroy it from 5 to 70 times as rapidly. He also found this rate to be increased under anærobic conditions but not with normal red cells.

As to the amount of glucose used up, Fulton [14] found this to be at the rate of 2·2 mg. in 1½ hours by 800 million parasites. This is equivalent to 18·4 mg. by $10^{10}$ parasites per hour or, reducing to molecular proportions, 1·02 mols. of glucose to 0·98 mols. of $O_2$, taking the figure for $O_2$ uptake we have previously given. It would therefore appear that, as with trypanosomes, the amount of glucose used up as compared to $O_2$ uptake is approximately unity.

Dr. Fulton and myself were unable to demonstrate by *p*H determinations that acid was formed in any appreciable amount. More recently Wendel [7] working with *P. knowlesi* found that if 400-500 mg. glucose be added to the suspension the *p*H falls progressively until all glucose is destroyed or until *p*H 5·5 is reached at which point respiration and glycolysis ceased. This author found approximately half of the destroyed glucose to be converted to lactic acid, the remainder being only partially oxidised.

Of a large number of sugars tested by Fulton with *P. knowlesi* only lævulose, maltose and mannose were found capable of giving an increase in $O_2$ uptake, but glycerol was utilised. A similar result in the main was obtained by Maier and Coggeshall.[5] They found succinate, fumarate, malate and citrate were not utilised, but fructose, mannose, as also glycerol and lactate were. Wendel [7] believed from his experiments that respiration was only indirectly dependent upon glycolysis, but was due to lactate metabolism.

The respiratory quotient for *P. knowlesi* was found by Christophers and Fulton [1] to approach unity (0·86). Velick [6] working with *P. cathemerium* obtained figures for the respiratory quotient in 9 birds varying between 0·61 and 0·94, the mean being 0·78. There is thus a marked

[14] Fulton, *Ann. Trop. Med. and Par.*, 1939, **33**, 217.

contrast in this respect to the trypanosomes *T. rhodesiense* and *T. equiperdum* where the quotient is in both cases under 0·2.

Time does not permit of these metabolic processes being discussed in further detail. In the main both in trypanosomes and malaria parasites the chief features are the utilisation of glucose, the taking up of oxygen and the formation of acid. But there are evidently some differences. These are even more strikingly apparent when one comes to consider the fundamental enzymic processes involved. Unfortunately these have as yet been very inadequately investigated.

We know that in the case of trypanosomes methylene blue is actively reduced in the Thunberg tube and presumably a hydrogenase system is involved. But the nature of the oxidising part of the system is as yet entirely unknown. Respiration is not inhibited by cyanide as is markedly the case with the malaria parasite showing that there is a different mechanism involved. In the case of trypanosomes deprivation from glucose for as short a period as 15 minutes completely does away with the power to restart $O_2$ uptake when glucose is later added. Very possibly this is due to the fact that deprivation of glucose leads to rapid lysis of the organisms. It is of interest in such experiments to see that deprivation of glucose has all the appearances of addition of some powerful toxic drug.

The malaria parasite probably also works through a hydrogenase system, but this so far has not been demonstrated owing to the fact that the presence of red cell itself reduces methylene blue in the Thunberg tube and attempts to investigate this point further have not been made. $O_2$ uptake by *P. knowlesi* is immediately and strongly inhibited by cyanide. Velick [6] working with *P. cathemerium* also found $O_2$ uptake to be completely inhibited by cyanide, and considers that it is likely therefore that the final stages of the biological oxidations in the parasite are catalysed by the cytochrome : cytochrome-oxidase system. Velick found demonstration of cytochrome-oxidase activity to be made difficult by the fact that the parasite must first be isolated from a system which itself contains the enzyme. But it was possible to demonstrate an increase in oxidase activity during progress of the developmental cycle. The method employed was the rate of oxidation of *p*-phenylene diamine. Though subject to interference by the presence of red cells the rate was found to augment as the parasite cycle advances, and at full maturity of the parasites it may be more than double the original rate when only young forms of the parasite were present. The activity, however, is not strictly parallel to the increase in $O_2$ uptake.

Keilin and Hartree [15] have made very clear the mechanism of working of cytochrome oxidase and hydrogenase systems. Hydrogenase specific for succinate leads to a transfer of $H_2$ provided a suitable source of $H_2$ is present. If methylene blue is present this is made use of and reduced in the process to the leucobase. Under normal conditions in the cell cytochrome *c* is present and is similarly reduced. But in the presence of indophenol-oxidase, which is specific for the reaction, the cytochrome is reoxidised from the atmosphere, thus acting as a "hydrogen carrier." Interference with any part of the chain would inhibit $O_2$ uptake. Where, as in some other systems, a co-enzyme or more than one co-enzyme enters into the reaction, any chemical or other destruction or blocking of such will also lead to inhibition of $O_2$ uptake. Inhibition of cell respiration is closely linked with lethality. We are thus brought to the importance of these enzymic activities in relation to the effects of lethal drugs.

Quinine and many compounds of this general type have long been known as inhibitors of enzyme activity and have been much studied from this point of view by Rona and Bloch [16] and others. It seems not at all improbable in view of this property that quinine and other antimalarial

[15] Keilin and Hartree, *Proc. Roy. Soc. B.*, 1937, **122**, 298.
[16] Rona and Bloch, *Biochem. Z.*, 1922, **128**, 169.

drugs of this general type may exert their effects on the enzymes of the parasite by acting in this way in the body. Added to the experimental flask even in minute amounts such compounds inhibit $O_2$ uptake by the parasite. They can be tested in this way and depending upon the concentrations at which they act can be given a coefficient of inhibition.[2] In their action thus *in vitro* there is often a suggestive relationship to therapeutic effectiveness. Thus among substances tested by Dr. Fulton and myself, quinine, atebrin and plasmoquin all actively inhibit $O_2$ uptake by *P. knowlesi*, and in much the same proportion as their therapeutic efficiency. A number of sulphonamide compounds have been tested in the same way by Coggeshall and Maier.[4]

That a test of this kind could be used as a short cut to determining the therapeutic effectiveness of different drugs could obviously not be claimed. At the same time it is not unreasonable to suppose that a direct effect *in vitro* of this kind may actually be an indication of the mode of action therapeutically. Also an absence of effect would appear to exclude action of a direct kind in the body. A good example of the latter is the contrast of the *in vitro* effect of pentavalent arsenical compounds on trypanosomes with that exerted in the body. It is generally agreed that these compounds are active therapeutically only because in the body they become changed slowly to the trivalent form. In conformity with this conclusion is the fact that whilst the pentavalent compounds do not act as inhibitors *in vitro*, the trivalent compounds are very powerfully inhibitive. Again Coggeshall and Maier [4] found sulphanilamide, sodium sulphathiazole and sodium sulphapyridine active *in vitro* in inhibiting $O_2$ uptake by *P. knowlesi*. "Prontosil" and sulphadiazine were found inactive. The latter is extremely insoluble. "Prontosil" is believed to be effective therapeutically only because it is split up in the body.

It is not, however, as a test, or short cut to looking for new effective compounds that investigation on these lines has its greatest interest or value. Could we determine what particular link in the cell respiratory mechanism was broken by a particular type of inhibitor and why, we should be in a much better position than we are at present to collaborate with the chemist in the synthesis of new compounds, assuming that it is actually in this way that antimalarial drugs act in the body.

It is improbable that every type of drug showing activity against the parasite acts in the same way, but among those which it may reasonably be supposed may act by being enzyme inhibitors are quinine and the other cinchona alkaloids, various derivatives more or less on the quinine model, compounds of the plasmoquin type, compounds related to atebrin and probably some less well known synthetic compounds with the same general chemical structure. The most outstanding feature of all these is that they are complex organic bases. Basic characters would seem in fact to be essential if substances of this type are to be effective in either role. Substitution of groups which eliminate the basic groups in the quinine molecule produces compounds inert against malaria. Schulemann [17] speaking of the search for a synthetic antimalarial compound which led to the discovery of plasmoquin said that until a basic component was included in the molecule no success was achieved. It seems not impossible that their basic make up is responsible for the effect of these compounds upon cell metabolism and possibly for their antimalarial activity.

But their basic make-up is comprised in their *pk* constants. These, along with solubility, determine almost all the reactions of any given base in the test tube, decide the extent to which it will act as a base at any given *p*H, control the proportions of free alkaloid and salts under any given conditions, fix the *p*H of its solutions, the proportion of monovalent and divalent salts and many other properties. Apart from solubility,

[17] Schulemann, *Proc. Roy. Soc. Med.*, 1932, **25**, 897.

and excluding molecular disruption, its *pk* constants make up almost in entirety its characters as a base. It seems therefore not unlikely that the inhibitor and antimalarial effects of such compounds are not so much a direct result of molecular structure as an indirect effect of this working through their basic make-up. If this be really so it has a very important bearing on the line of approach to synthesis of new antimalarial compounds.

## Conclusions.

1. The respiratory metabolism of *P. knowlesi*, *P. inui*, *P. cynomologi*, *P. cathemerium* and *P. lophuræ* is characterised by utilisation of glucose, the taking up of $O_2$ in approximately equal molecular proportion and formation of lactic acid. According to Wendel [7] the direct substrate is lactate, glucose being utilised only indirectly.

2. The process appears to depend upon a hydrogenase-cytochrome-oxidase system, but has as yet been imperfectly investigated.

3. Conspicuous among agents inhibiting such metabolism are basic alkaloid or alkaloidal-like substances including quinine, plasmoquin and atebrin with other antimalarial compounds of this type. Investigation of such inhibitor effect is important both as an *in vitro* test and in fundamental research upon the mechanism of action of antimalarial drugs.

4. The basic make-up of these compounds expressed by their *pk* constants may be more concerned in such effects than their actual molecular structure.

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*Cambridge.*

## *GENERAL DISCUSSION.*

**Dr. H. R. Ing** (*Oxford*) said : If the important feature of the inhibitory activity of quinine, plasmoquin and atebrin are the *pk* values of the two basic groups, and if the molecular structure of antimalarials of this type is only important in the sense that it determines these *pk* values, it follows that any organic molecule containing two basic groups with the right *pk* values should possess inhibitory activity on the malarial parasites. Such a view would open an enormous field to the organic chemist, but it seems more probable that the *pk* values constitute limiting factors on molecular structures which *on other grounds* possess antimalarial properties.

| Alkaloid. | $pk_1$. | $pk_2$. |
|---|---|---|
| Quinine (*a*) | 5·97 | 9·70 |
| Quinine (*b*) | 5·70 | 9·85 |
| Quinidine (*a*) | 5·43 | 10·00 |
| Cinchonine (*a*) | 5·85 | 9·92 |
| Cinchonidine (*a*) | 5·80 | 10·03 |
| Nicotine (*a*) | 6·16 | 10·96 |
| Atebrin (*b*) | 3·88 | 6·47 |
| Plasmoquin (*c*) | 3·93 | 10·51 |

(*a*) Kolthoff, *Biochem. Z.*, 1925, **162**, 289 ; (*b*) Christophers, *Ann. Trop. Med.* 1937, **31**, 43 ; (*c*) *idem.*, *ibid.*, 1940, **34**, 1.

It is worth noting in this connection that the apparent *pk* values of nicotine are closer to those of the quinine alkaloids than those of atebrin and plasmoquin.

**Sir Rickard Christophers** (*Cambridge*), in answer to Dr. Ing, said : The idea that if we knew what were suitable *pk* values for a base the molecular chemist might help in the search for new antimalarial compounds was certainly in my mind. A trial on these lines was certainly worth making. Some of the alkaloids whose basic make-up was suitable might quite well be effective against malaria, but this could never be tried be-

cause they were highly poisonous due to other features in their molecular structure.

**Prof. J. H. Gaddum** (*Edinburgh*) said : This kind of experiment is apt to give misleading results if it is necessary to use high concentrations of drugs to get an effect. Were the concentrations actually used of the same order as those present in the blood when the drugs are used therapeutically ?

**Sir R. Christophers,** *in reply to Prof. Gaddam,* said : The concentrations used in our inhibition experiments were often quite of the order in which drugs might occur in the body. In an experiment lasting a relatively short time, however, higher concentrations might be necessary to give results than might be effective in many hours in the body.

**Mr. F. Hawking** (*Hampstead*) said that Sir Rickard Christophers' conception that antimalarials are strongly basic does not conform with the fact that sulphonamides, which have an antimalarial action, are approximately neutral, or faintly acid in solution.

**Prof. Gaddum** said : This technique seems to offer a good opportunity of finding out more about the mechanism by the study of antagonists. Has any search been made for substances which might antagonise the action of drugs in experiments of this kind on malaria ?

**Sir R. Christophers,** in reply, said : It is very improbable that all drugs having an effect in malaria work through the same mechanism. I was careful in my paper to speak of drugs of the quinine, atebrin, plasmoquin type. One would expect an arsenical compound or a sulphonamide to work in a different manner.

**Dr. H. Hurst** (*Cambridge*) said : One of the chief difficulties in the interpretation of antimalarial toxicological data lies in the lack of precise knowledge as to the actual site of drug interaction in the parasite. The experimental evidence suggests that the drug-receptor groups in the parasite enzyme systems are separated from the external drug phase by a protective cell wall which has a lipo-protein mosaic ultrastructure. In systems of this type, selective drug interaction with the cell wall may obscure more specific interaction with the enzyme system, and this may account for the lack of consistency which is apparent in attempts to correlate the antimalarial activity of a drug with expressions of chemical reactivity involving specific pharmacodynamic groups in the drug molecule and drug-receptor combinations. Theories of drug action involving highly specific drug-enzyme-substrate associations are only valid when the disturbing influence of a selective *rate* of drug access to the site of action has been evaluated. The decrease in drug reactivity with the elimination of the basic groups in alkaloids such as quinine is consistent with a corresponding decrease in drug mobility along the network of functional lipo-protein interfaces of the cell wall framework. The basic groups of the drug molecules appear to exert a "carrier" action on the drug molecules which involves polar interaction with the protein components of the cell wall associated with selective penetration or perhaps dispersion of this phase. In this way, the accessibility of the pharmacodynamic portion of the drug molecule which brings about inhibition of enzyme activity may be greatly influenced. The possibility also exists, however, that change in the basic portion of the drug molecule may influence to some extent drug reactivity at the ultimate site of action in the parasite. Similar results have been with insecticidal drugs, where reactivity involves a prior penetration through a relatively macroscopic lipo-protein framework of the insect cuticle.

**Sir R. Christophers,** in reply, said : So far as I am aware, nothing is known regarding the distribution of enzymes in the malaria parasites or the intimate surface structure of these organisms.

# THE BLOOD-BRAIN BARRIER AND CEREBRO-SPINAL FLUID, IN RELATION TO THE EFFICACY OF SLEEPING-SICKNESS DRUGS.

By E. M. Lourie.

(*Medical Research Council and Liverpool School of Tropical Medicine.*)

*Received 9th August*, 1943.

Sleeping sickness, or human trypanosomiasis of Africa, may be regarded as a disease of essentially two stages. In the first the infection is practically limited to the blood and the lymphatic system. After a variable time, during which the tissue-spaces of various organs may or may not be invaded, there follows the second and very serious stage, in which some of the trypanosomes have found their way into the substance of the brain. A drug intended as a remedy for sleeping sickness has therefore a considerably more exacting task to perform in the second than in the first stage of the disease, for the reason that those parasites which have penetrated beyond the blood-stream into intimate relationship with the brain-cells are, in their new situation, relatively sheltered from direct impact with trypanocidal agents circulating in the blood. The drug or its metabolite has now to find its way, or to exercise its influence, beyond the blood-brain barrier. It is accordingly important to understand the nature of this barrier and to define the extent and conditions of its resistance to the passage of trypanocides into the extravascular portions of the brain. These are, however, matters about which much remains yet to be explained, and the field should be a most fruitful one for the combined efforts of biologists, chemists, and physical chemists. I intend here to present some facts and opinions as a basis for discussion and further work on the subject.

## Localisation of the Blood-brain Barrier, and its Relationship to the Cerebro-spinal Fluid.

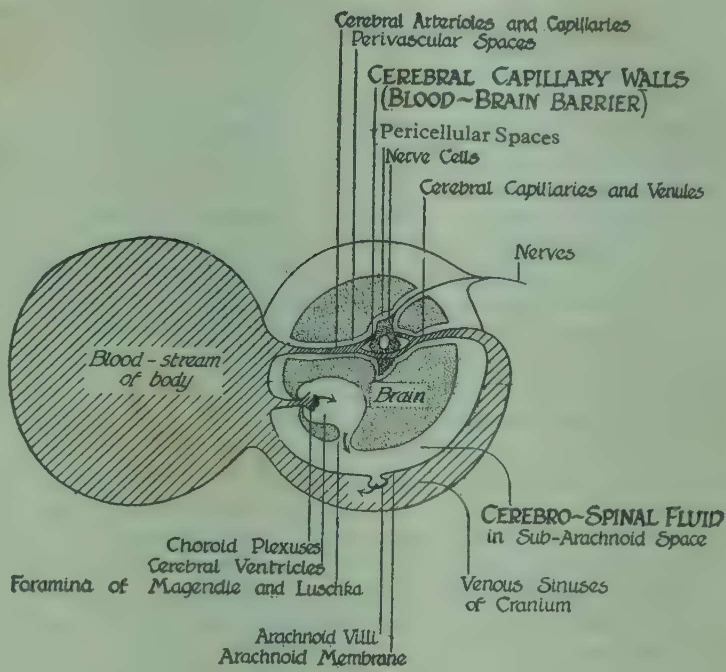
The term "blood-brain barrier" (*barrière hémato-encéphalique*) was introduced by Stern,[1] and its lack of anatomical precision indicates the uncertainty that has existed as to its exact localisation, and also that its functions are probably to be regarded as not being exercised by a single strictly delimited organ or system. In fact Stern suggested that the term might be considered not in a purely anatomical sense, but rather as applicable to the mechanism which exercises a selective control over the passage of substances from blood to brain. It is, however, convenient to follow the practice of subsequent writers who have used the term as much in an anatomical as in a functional connotation. In the drawing (p. 341) I have attempted to show diagrammatically the anatomical relationships of the organs and fluids concerned.

It seems certain that the blood-brain barrier is mainly localised in the walls of the cerebral capillaries (Spatz[2]; Friedemann[3,4]); that is to say the passage of substances from blood to brain-cells presumably occurs, for the greater part, directly from the cerebral capillaries through the capillary endothelium into the intercommunicating perivascular and

[1] Stern, *Schweitz. Arch. Neurol. Psychiat.*, 1921, **8**, 215.
[2] Spatz, *Arch. f. Psychiat.*, 1933, **101**, 267.
[3] Friedemann, *Lancet*, 1934, **1**, 719, 775.
[4] Friedemann, *Physiol Rev.*, 1942, **22**, 126.

pericellular spaces surrounding the brain-cells. There is little doubt that this view is more tenable than theories (*e.g.* Stern ;[5] Monakow[6]) criticised by Spatz and by Friedemann, which regard the choroid plexus

Diagram showing relationship of the blood-brain barrier to the cerebro-spinal fluid. Source and circulation of the latter are mainly as indicated by the arrows, but some is believed to be formed also in the perivascular spaces and poured into the subarachnoid space.




or the meningeal vessels as essential parts of the blood-brain barrier, and according to which, therefore, substances finding their way from the blood to the immediate neighbourhood of the brain-cells must necessarily pass through the cerebro-spinal fluid.

## The Need of a Technique for determining the Ability of Trypanocides to appear beyond the Blood-brain Barrier.

It is obvious that, in the purposive search in the laboratory for compounds worthy of trial against sleeping-sickness, it would be of very considerable value to have some simple technique which would indicate whether a drug introduced into the blood-stream is capable of appearing, in trypanocidal form, beyond the blood-brain barrier, in the perivascular and pericellular spaces, where it would come into direct contact with trypanosomes in second stage sleeping-sickness. The classical method of selecting potential sleeping-sickness remedies on the basis of their therapeutic effect on the acute septicæmic trypanosome infections of mice clearly does not suffice. This is strikingly illustrated by the case of Antrypol (Bayer 205, Germanin).* In the mouse this compound has an exceptionally high therapeutic index (Haendel and Joetten ; Meyer and Zeiss ; Wenyon ; Yorke, Murgatroyd and Hawking[7, 8, 9, 10]) far outstripping tryparsamide, yet it has proved considerably inferior to the latter compound in second stage sleeping-sickness. The inadequacy of

[5] Stern, *Schweitz. Med. Woch.*, 1923, **53**, 792.
[6] v. Monakow, *Schweitz. Arch. Neurol. Psychiat.*, 1921, 8, 233.

* The formulæ of compounds referred to in this paper are given in an Appendix.

[7] Haendel and Joetten, *Berl. klin. Wschr.*, 1920, **57**, 821.
[8] Mayer and Zeiss, *Arch. Schiffs- u. Tropenhyg.*, 1920, **24**, 257.
[9] Wenyon, *Brit. med. J.*, 1921, **2**, 746.
[10] Yorke, Murgatroyd and Hawking, *Ann. trop. Med. Parasit.*, 1931, **25**, 313.

tests on infections in mice in the preliminary laboratory evaluation of a drug was well realised by Brown and Pearce [11] in their studies on tryparsamide, prior to its actual use against sleeping-sickness. They stressed the importance, in such preliminary work, of making some determination of the drug's power of penetration into the extravascular tissues, and this they considered could suitably be done merely by measuring its therapeutic potency in the chronic infections of rabbits, in which trypanosomes are to be found not only in the blood but also in tissue-spaces in various parts of the body. They found that in mouse infections by *Trypanosoma brucei*, *T. gambiense* and *T. equinum* the therapeutic index of tryparsamide could be described as no more than "fairly good but by no means equal to that of a number of well known substances, such as arsphenamin and neo-arsphenamin, and it is distinctly inferior to that of arsenophenyl-glycine or even arsacetin." However, in well-developed rabbit infections they were satisfied of the superiority of tryparsamide for the reason that, unlike the other arsenicals, it was curative in the same dose per unit bodyweight as for the acute infections of mice. Interpreting this as evidence that the drug possessed a high degree of what they termed "therapeutic penetrability," Brown and Pearce regarded it as the one feature more than any other which justified its trial in naturally occurring trypanosomiasis. However, in spite of the example of tryparsamide, experience has shown that we still cannot gain a sufficient idea of the potential usefulness of a compound in late sleeping-sickness by the mere practice of supplementing tests on the acute mouse septicæmia by further tests on the chronic rabbit infection. This is well shown in the results obtained with drugs which have recently been tested against sleeping-sickness in Africa, namely the arsenical compound Neocryl, and the diamidines, Stilbamidine, Pentamidine and Propamidine. Neocryl had previously been found by Yorke and Murgatroyd [12] to be more effective than tryparsamide in rabbit infections, but it has since proved to be less so in actual late cases of sleeping-sickness (Acres [13]). Similarly the diamidines mentioned were found to be superior to tryparsamide in rabbit infections (Lourie and Yorke [14]) but not in late sleeping-sickness (Harding, Bowesman, Lourie, Lawson [15, 16, 17, 18]).

It is clear that we need a more direct means of deciding whether a drug has the power of appearing in trypanocidal form beyond the blood-brain barrier. The use of dyes, as in the pioneer work of Ehrlich,[19] is of only limited value in this connection. Thus, if the brain-cells are stained after intravenous or subcutaneous injection of a trypanocidal dye, one may indeed infer that the blood-brain barrier is permeable to that particular compound, but no safe assumption may be made on the important question of its concentration in the perivascular and pericellular spaces of the brain. If on the other hand there is no staining of the brain-cells, this does not necessarily mean that the dye has not reached the tissue spaces, for the explanation may simply be that the brain-cells lack an affinity for the dye (King [20]). In the case of the arsenical drugs in general one may of course estimate the amount of arsenic which lodges in the brain after an intravenous injection, and if this proves to be more than can be accounted for by the blood present in the specimen examined, one may assume that arsenic has passed the barrier (if it has not been

[11] Brown and Pearce, *J. Amer. med. Ass.*, 1924, **82**, 5.
[12] Yorke and Murgatroyd, *Brit. med. J.*, 1936, **1**, 1042.
[13] Acres, *Trans. R. Soc. trop. Med. Hyg.*, 1940, **34**, 281.
[14] Lourie and Yorke, *Ann. trop. Med. Parasit.*, 1939, **33**, 289.
[15] Harding, *ibid.*, 1940, **34**, 101.
[16] Bowesman, *ibid.*, **34**, 217.
[17] Lourie, *ibid.*, 1942, **36**, 113.
[18] Lawson, *Lancet*, 1942, **2**, 480.
[19] Ehrlich, *Therap. Monatsh.*, 1887, **1**, 88.
[20] King, *Arch. Neurol. Psychiat.*, 1939, **41**, 51.

disproportionately adsorbed to the walls of the blood-vessels). But this will give no indication as to whether the form which it has assumed beyond the barrier, in the perivascular and pericellular spaces, is or is not trypanocidal. One would wish, if it were possible, to obtain samples of the fluid in these spaces for tests of trypanocidal activity but it seems to be technically quite an unattainable ideal adequately to tap these microscopical channels.

It has frequently been assumed that the power which a drug possesses of penetrating the blood-brain barrier after introduction into the blood-stream may be judged by its subsequent identification in the cerebro-spinal fluid, *i.e.* that if a compound appears in the cerebro-spinal fluid it must therefore be capable of appearing in the tissue spaces of the brain. This is, however, an unjustifiable assumption, when one considers the commonly accepted theory of the origin and circulation of the cerebro-spinal fluid in the cranial cavity (see diagram). The fluid is believed to be formed mainly by the choroid plexuses in the cerebral ventricles ; it then passes through the Foramina of Magendie and Luschka into the subarachnoid space, whence it is absorbed into the blood-stream mainly *via* the arachnoid villi in the cranial venous sinuses. It may be seen that this circuit does not anywhere traverse the brain-tissue, and it follows therefore that if, after administration say intravenously or by mouth, a drug be detected in the cerebro-spinal fluid, this is no reliable indication that it has penetrated the blood-brain barrier, or that it will do so. One must, however, not lose sight of the fact that, although the bulk of the cerebro-spinal fluid is believed, on very good evidence, to be produced by the choroid plexuses, it seems probable that a small quota is also contributed by fluid reaching the subarachnoid space directly from the perivascular spaces of the brain (Weed [21]). While it remains true, therefore, that a compound found in the cerebro-spinal fluid has not necessarily traversed the tissue spaces of the brain, yet it may to some slight extent have done so. There can likewise be no unequivocal interpretation of a failure to detect the compound in the cerebro-spinal fluid. While this might well mean that it had not passed the blood-brain barrier, there does remain the possibility that it may in fact have been in effective concentration in the minute perivascular spaces of the brain, but on reaching the sub-arachnoid space it may have become so diluted, in the bulk of fluid formed at the choroid plexuses, as to be no longer detectable.

The position seems to be then that there is no unexceptionable means at our disposal of directly determining whether a drug has or has not the power of appearing, in trypanocidal form, in the tissue spaces of the brain. Probably our nearest approach to the decision would lie in examination of the cerebro-spinal fluid, although on theoretical grounds this method remains subject to serious doubts and questions. There is, however, the following further consideration in favour of testing the penetrating powers of potential trypanocides into the cerebro-spinal fluid. The simple statement that in second stage sleeping-sickness some of the trypanosomes find their way from the blood to the tissue-spaces of the brain does not necessarily imply that they always do so exclusively by direct passage through the capillary walls. Although characteristically they are easily demonstrable in the cerebro-spinal fluid in late sleeping-sickness, there are, in fact, very few recorded observations of trypanosomes actually seen in sections of brain, and there is no certainty as to the usual route by which they pass from the blood to the extravascular portions of the brain. They have been found in the latter situations in man by Stevenson,[22, 23] and in experimental animals by Wolbach and Binger,[24]

[21] Weed, *Physiol. Rev.*, 1922, **2**, 171.
[22] Stevenson, *Trans. R. Soc. trop. Med. Hyg.*, 1922, **16**, 135.
[23] Stevenson, *ibid.*, 1923, **16**, 384.
[24] Wolbach and Binger, *J. med. Res.*, 1912, **27**, (New Series **22**), 83.

Stevenson,[23] Peruzzi,[25] and Hoeppli and Regendanz.[26] Wolbach and Binger found them to be present in considerable numbers not only in the perivascular spaces and in the perivascular cellular infiltrations typically associated with brain-involvement in trypanosomiasis, but also actually in the walls of small blood vessels and capillaries. This suggests that, as has often been assumed, the trypanosomes do pass directly from blood-capillaries to the perivascular spaces. However, Stevenson, Peruzzi, and Hoeppli and Regendanz found them in masses in the choroid plexuses, and it is likely that in most of the cases of sleeping sickness in which trypanosomes are present in the cerebro-spinal fluid the organisms in this situation derive immediately from the choroid plexus. If this be so, then trypanosomes which invade the brain, in second stage sleeping-sickness, may do so, at least in some cases, *via* the ventricular or subarachnoid cerebro-spinal fluid, rather than directly through the walls of the cerebral capillaries. Quite independently of its powers of penetrating the cerebral capillary walls, therefore, a drug which can attack trypanosomes in the ventricular and subarachnoid spaces should be preferred, on theoretical grounds, to one which acts only within the blood-stream.

Trypanosomes which pass from the cerebro-spinal fluid into the brain substance probably do so by journeying up the perivascular and pericellular spaces (see diagram) and no doubt their motile powers would facilitate penetration in what might otherwise be an unlikely direction. The idea that trypanosomes are possibly capable of passing from the circulating blood to the brain *via* the cerebro-spinal fluid raises again the question whether drugs may not, after all, follow a similar route, in accordance with the discredited theories of Stern and Monakow,[5, 6] mentioned above. In other words, if a compound be shown to have reached the cerebro-spinal fluid from the blood-stream, may we expect it to flow or diffuse into the brain along the perivascular and other similar microscopical channels? It is perhaps reasonable to expect such a diffusion particularly if the concentration of the compound in the cranial venous sinuses be considerably higher than in the cerebro-spinal fluid, as would certainly be the case after the intravenous injection of some drugs. It is generally believed, however, that the actual flow of fluid in the perivascular spaces is normally in the outward direction, towards the surface of the brain. In this connection it is worth mentioning, in passing, that there is evidence (*e.g.* Weed and McKibben; Foley [27, 28]), that the normal direction of flow in these spaces can be reversed by the osmotic effects of the intravenous injection of strongly hypertonic solutions of sodium chloride. Weed and McKibben describe the process as "the dislocation of a considerable quantity of the cerebro-spinal fluid into the nervous system." In the light of this it might be worth while to explore the therapeutic potentialities of combining treatment by drugs known to be capable of penetrating into the cerebro-spinal fluid with the intravenous injection of strongly hypertonic sodium chloride solutions, in advanced sleeping-sickness.

### Examination of Trypanocidal Power of the Cerebro-spinal Fluid after Intravenous Treatment.

The trypanocidal power of cerebro-spinal fluid after intravenous injection of various arsenicals has been tested by Voegtlin *et al.*[29] and by

[25] Peruzzi, "Final Report of the League of Nations International Commission on Human Trypanosomiasis," Geneva, 1928.

[26] Hoeppli and Regendanz, *Arch. Schiffs- u. Tropenhyg.*, 1930, **34**, 1 and 67.

[27] Weed and McKibben, *Amer. J. Physiol.*, 1919, **48**, 531.

[28] Foley, *Arch. Neurol. Psychiat.*, 1921, **5**, 744.

[29] Voegtlin, Smith, Dyer and Thompson, *Publ. Hlth. Rep.*, 1923, **38**, 1003.

Hawking *et al.*[30, 31, 32] Voegtlin used rabbits, and his method was to trephine the skull and then to inject trypanosomes into the subarachnoid space and the drug into the marginal ear vein. After 24 hours the animal was killed, the trephine opening enlarged, and cerebro-spinal fluid withdrawn and examined for the presence of trypanosomes. Hawking used human subjects, and he tested the trypanocidal power of the cerebro-spinal fluid *in vitro*, according to the technique of Yorke, Adams and Murgatroyd.[33] The method used by Hawking is simpler than that of Voegtlin, but there are obvious difficulties, as well as certain technical disadvantages, in the use of human subjects for large-scale routine preliminary tests of cerebro-spinal fluid activity, in the search for new remedies for sleeping-sickness. With Dr. H. O. J. Collier, in work which is as yet unpublished, I have returned to the use of rabbits, but our technique does not involve the trephining operations which Voegtlin found necessary, and, like Hawking, we estimate the trypanocidal activity of the cerebro-spinal fluid by tests *in vitro*.

Of the compounds examined by Voegtlin and by Hawking and their co-workers, certain pentavalent aromatic arsonates, notably tryparsamide and orsanine were found to render the cerebro-spinal fluid trypanocidal after intravenous injection, while others, such as neocryl, were deficient in this property. These results are in line with the proved therapeutic value of tryparsamide and orsanine, and the ineffectiveness of neocryl in advanced sleeping-sickness. Among the arsenobenzene derivatives, arsphenamine and neoarsphenamine imparted only inconspicuous trypanocidal powers to the cerebro-spinal fluid, but sulpharsphenamine did not fall far short of tryparsamide in this respect. Collier and I have confirmed, in our tests *in vitro*, that tryparsamide renders the cerebro-spinal fluid of rabbits trypanocidal; the trivalent derivative of tryparsamide, reduced tryparsamide thioglycollate, lacks this property. We have also found neoarsphenamine to be far more effective than did Voegtlin and Hawking, in conferring trypanocidal powers on the cerebro-spinal fluid, and this, together with Voegtlin's and Hawking's finding in regard to sulpharsphenamine, mentioned above, suggests that the potentialities of the arsenobenzene derivatives in the chemotherapy of sleeping-sickness have not yet been fully explored.

## Factors Determining the Ability of Substances (*a*) to Penetrate the Blood-brain Barrier, and (*b*) to Appear in the Cerebro-spinal Fluid.

The factors which determine ability of compounds to penetrate the blood-brain barrier are not well understood, and the literature on the subject is somewhat confused. Friedemann[3, 4] has reviewed the matter, and he states that as far as non-polar substances are concerned there is little doubt that lipoid solubility or surface activity play an important part. However, among polar substances (with which we are mainly concerned), Friedemann concludes that molecular size, lipoid solubility and diffusibility are only of minor consequence, the important factors being the electro-chemical properties of the substances concerned. He claims that, in the toxins and drugs investigated, ability to penetrate the blood-brain barrier is associated with a positive or no charge at the *p*H of blood, while inability to penetrate the barrier is associated with a negative charge.

Wittgenstein and Krebs[34] studied the penetration of substances from

[30] Hawking, Hennelly and Quastel, *J. Pharmacol.*, 1936, **59**, 157.
[31] Hawking, Hennelly and Wales, *ibid.*, 1938, **64**, 146.
[32] Hawking, *Trans. R. Soc. trop. Med. Hyg.*, 1940, **34**, 269.
[33] Yorke, Adams and Murgatroyd, *Ann. trop. Med. Parasit.*, 1929, **23**, 501.
[34] Wittgenstein and Krebs, *Z. Ges. exp. Med.*, 1926, **49**, 553.

blood to cerebro-spinal fluid, and the factors which they found to be associated with this passage are at variance, in several particulars, with those which Friedemann regards as determining penetration of the blood-barrier. Like Friedemann, they attached great significance to electro-chemical properties, but unlike his conclusion in regard to penetration of the blood-brain barrier, their finding was that among diffusible substances it is the negatively charged acid compounds which penetrate into the cerebro-spinal fluid, while the positively charged basic substances fail to do so. Penetration into the cerebro-spinal fluid was also believed to be partly determined by diffusibility, or degree of dispersion, since substances which are of a colloidal nature, even though they carry a negative charge, do not appear in the cerebro-spinal fluid. The conclusions of Wittgenstein and Krebs seem to be broadly in harmony with known facts in regard to the powers of penetration into the cerebro-spinal fluid of drugs effective against trypanosome infections, though it remains for further work to establish the precise extent of this agreement. Thus, tryparsamide, which has the power of penetrating into the cerebro-spinal fluid, is the sodium salt of an acid, and probably circulates in the blood, to some extent at least, as a negatively charged ion. Neoarsphenamine presumably also circulates in the anionic state, and sulpharsphenamine, which is said to penetrate more readily into the cerebro-spinal fluid than neoarsphenamine, is probably still more electronegative in character than that compound, as an effect of its rather strongly acid radicals. The diamidines, which appear to be incapable of reaching the cerebro-spinal fluid, are salts of strong bases and undergo a high degree of ionisation in dilute solution.[35] Antrypol, which has only slight powers of penetration into the cerebro-spinal fluid, is an acidic compound, presumably circulating in the blood, in part, as an anion, but it has a very large molecule and probably circulates in a semi-colloidal form, or, as believed by Mayer and Zeiss,[36] bound to plasma proteins.

In attempting to correlate the electro-chemical properties of substances with their powers of penetrating vital membranes, it must be remembered that numerous complicating (or related) factors operate *in vivo*. There are for example different rates of disappearance from the blood-stream, and different rates of excretion. There is also the factor of different degrees of toxicity. In general, electronegative are much less toxic than electropositive substances, and they remain in the circulation for a longer time. Accordingly, since they can be introduced into the circulation in greater amount, and do not disappear as rapidly, they have to that extent a better opportunity of penetrating vital membranes. However, their lower toxicity for the host reflects also, in general, a lower lethality to the trypanosome; mere ability of a negatively charged substance to appear in the cerebro-spinal fluid is valueless for our purpose if unaccompanied by trypanocidal properties. With these considerations in mind the case of drugs of the tryparsamide type is perhaps highly significant. Tryparsamide is relatively non-toxic to the host and has practically no direct trypanocidal action. These features may, according to the foregoing, be considered as related to its electronegative charge, by virtue of which also it gains admission to the cerebro-spinal fluid. It is then reduced to the corresponding trivalent compound, this involving a radical alteration in the charge carried, together with the emergence of a highly trypanocidal property. The effect is, therefore, that a substance (reduced tryparsamide) which is extremely lethal to trypanosomes but which is unable, by its own nature, to penetrate into the cerebro-spinal fluid is nevertheless rendered available for action in that situation.

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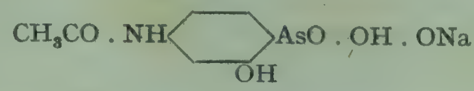
*Pembroke Place, Liverpool* 3.

[35] Henry and Grindley, *Ann. trop. Med. Parasit.*, 1942, **36**, 102.
[36] Mayer and Zeiss, *Arch. Schiffs- u. Tropenhyg.*, 1921, **25**, 259.

## APPENDIX.—Chemical Formulæ

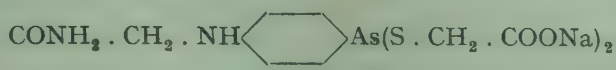
Tryparsamide


Orsanine

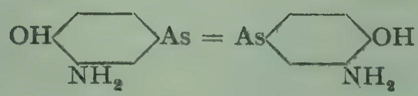



Neocryl

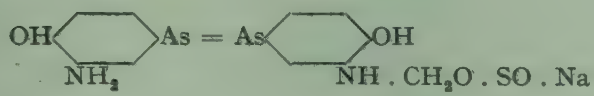

Reduced tryparsamide thioglycollate



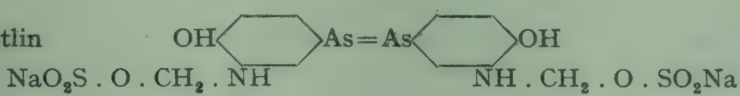

Arsphenamine



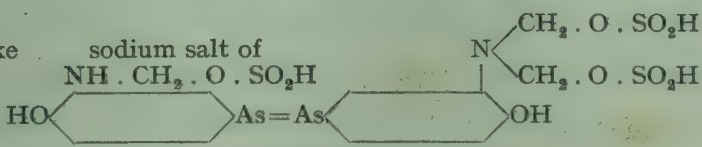

Neoarsphenamine




Sulpharsphenamine
(according to Voegtlin and Johnson [37])

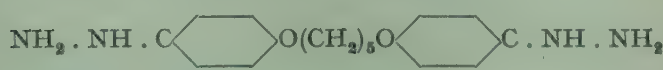



(according to Dyke and King [38])

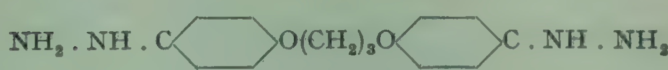



Stilbamidine


Pentamidine




Propamidine

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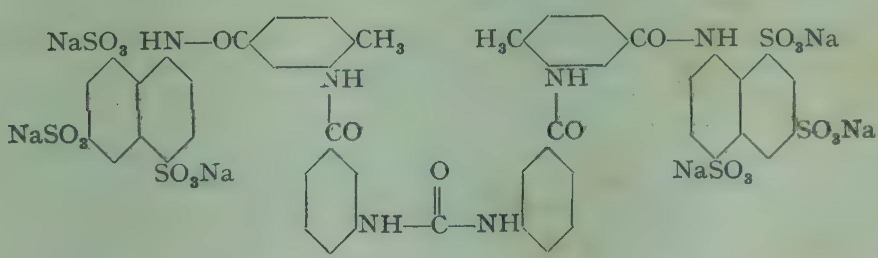



[37] Voegtlin and Johnson, *J. Am. Chem. Soc.*, 1922, 44, 2573.
[38] Dyke and King, *J. Chem. Soc.*, 1935, 805.

*GENERAL DISCUSSION*

**Dr. E. M. Lourie** (*Liverpool*), in introducing his paper, said: Before offering my contribution to this Discussion, I would like to associate myself with the handsome and very fitting tribute which Sir Henry Dale has paid to the memory of Warrington Yorke.

It was my great privilege to know and to work intimately with Yorke for many years until the time of his death. He endeared himself as a wise chief, a generous colleague, and a loyal friend.

He will be sadly missed by many, not only because of his outstanding achievements in the advancement of Science, but also because of the exceptional person that he was.

**Dr. H. McIlwain** (*Sheffield*) said: Dr. Lourie's communication, in conjunction with my own, emphasises the complexity, both chemical and structural, of the system involved in chemotherapy, and the care which should be exercised before conclusions respecting the mode of action of an agent are drawn from its differing activities under different conditions. It is an indication of the immaturity of chemotherapy that many investigators do not discriminate between the chemical (including biochemical) and structural factors before concluding that one or the other is responsible for variations in chemotherapeutic activity. Thus, activity or inactivity in different hosts may depend upon varying concentrations of antagonists, varying metabolism of the drug or antagonists, varying location of the infection, differences in natural defences, and other circumstances. These have rarely been fully explored, though many opinions have been expressed from partial investigations which in reality are inadequate to show whether an active agent has reached a given site; whether, when reaching it, the agent has been inactivated by antagonists or metabolism; or whether the differences lie in other activities of the parasite and host.

# EFFECTS OF NARCOTICS AND BENZEDRINE ON METABOLIC PROCESSES IN THE CENTRAL NERVOUS SYSTEM.

By J. H. Quastel.

*Received 16th September*, 1943.

Narcotics include a large variety of structural types such as hydrocarbons, alcohols, ethers, urethanes, sulphones, amides, etc., and it is evident that their common characteristic of inducing narcosis in animals must depend upon certain physicochemical characters which they have in common rather than upon the possession of any special chemical constitution. The Overton-Meyer theory suggests one important factor in the determination of narcotic activity, *i.e.*, the relative solubilities in the lipoid and non-lipoid constituents of the cell or cell surfaces. It is clear, however, that if the non-polar-polar character of a substance determines, or partly determines, its entry into a cell, or its orientation at a surface, it gives no obvious clue to the mechanism of action of the narcotic.

The narcotics as a class are, biochemically, inert molecules undergoing little or no change so far as is known in presence of the living cell. It is reasonable to suppose that their pharmacological activities are directly related to their surface activities.

If it can be shown that the narcotics are associated with a metabolic process in the cell and more particularly with some specific component involved in this process, an advance will have been taken in relating

narcosis to cell chemistry, and in throwing more light on the manner in which narcotics exert their physiological effects.

It has long been known that narcotics as a general rule inhibit enzymic and respiratory processes but until recently there has been reluctance to associate narcosis with a suppression of oxidative events. This has been partly due to the fact that the quantities of narcotics required to induce narcosis in an animal are usually of a far smaller order than those required to inhibit enzymic reactions. Moreover, there has not been until recently any clear evidence that during narcosis in an animal there is a fall in respiratory activity in the nervous system upon which the narcotics might be expected to exert their greatest effects.

McClure *et al.* [1] have, however, demonstrated the existence of anoxia in the central nervous system during the anæsthesia brought about by barbiturates and other narcotics, and according to Shaw *et al.* [2] ether anæsthesia is associated with a decrease in the difference between the oxygen contents of arterial and venous bloods. Dameshek *et al.*[3] have shown that in the human subject under the influence of amytal there is a small but definite inhibition of oxygen uptake and dextrose utilisation by the brain.

Very many facts point to the profound influence of brain oxidations on its functional activity. Moreover it has been clearly established that glucose is the main substrate of the brain in the living animal, and that the oxygen utilised by the brain is mainly concerned with combustion of glucose supplied by the blood (for evidence see [4]). A deprivation of glucose from the brain has as dire physiological effects as a deprivation of oxygen.

Some idea of the vulnerability of the brain to oxygen lack may be gained by the following facts. An interruption of cerebral circulation for 6 to 8 seconds will produce loss of consciousness. The usual action potentials in the cat's cortex are obliterated by a cerebral anæmia of 20 seconds.[5] Oxygen lack in dogs for longer than 5 minutes results in cessation of cerebral functions.[6] Unconsciousness will supervene if the oxygen supply to the brain is suddenly reduced to such an extent that oxygen saturation of the blood in the internal jugular vein falls to 24 % or less. Consciousness is maintained if the oxygen saturation value is 30 % or more.[7] Brain potentials are abolished in anoxæmia.[8]

In hypoglycæmia due to insulin administration there is a marked change in nature of cortical potentials, and electrical activity may cease if the hypoglycæmia is prolonged.[9] The frequency of cortical potentials becomes lower with fall of blood sugar and at low levels of blood sugar the waves may disappear.[10] The administration of glucose results in a restoration of the original rhythm after an interval depending on the severity and duration of the preceding hypoglycæmia.

Thus both oxygen and glucose lack lead to a slowing or abolition of cortical potentials. Gibbs *et al.* have now shown [11] that the administration of luminal brings about a slowing in the rate of cortical waves.

The physiological facts point to a very high degree of dependence of mental function on the maintenance of oxygen and glucose supply to the

[1] McClure, Hartmann, Schnedorf and Schelling, *Ann. Surg.*, 1939, **110**, 836.
[2] Shaw, Steele and Lamb, *Arch. Surg.*, 1937, **35**, 1.
[3] Dameshek, Myerson and Loman, *Am. J. Psychiat.*, 1934, **91**, 113.
[4] Quastel, *Physiol. Rev.*, 1939, **19**, 135.
[5] Simpson and Darbyshire, *Amer. J. Physiol.*, 1934, **109**, 99.
[6] Heymans and Bouckaert, *Compt. Rend. Soc. Biol.*, 1935, **119**, 324.
[7] Lennox, Gibbs and Gibbs, *Arch. Neurol. Psychiat.*, 1935, **34**, 1001.
[8] Davis, Davis and Thomson, *Amer. J. Physiol.*, 1938, **123**, 51; Hoagland, *ibid.*, 1938, **123**, 102.
[9] Hoagland, Rubin and Cameron, *ibid.*, 1937, **120**, 559.
[10] Maddock, Hawkins and Holmes, *ibid.*, 1939,
[11] Gibbs, Gibbs, and Lennox, *Tr. Amer. J. Neurol.*, 1937, **63**, 129.

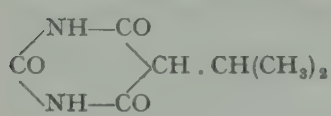
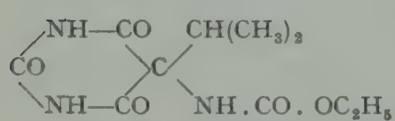
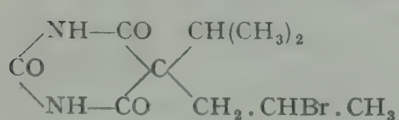
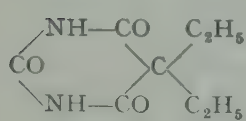
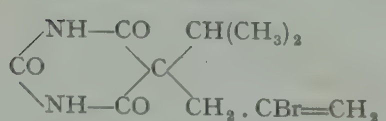
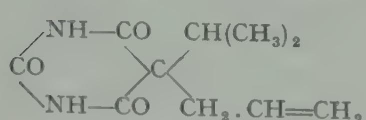
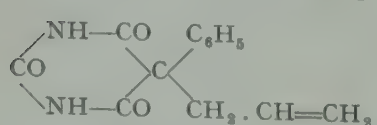
central nervous system. An interference with the respiratory activity of the nervous system by the action of a drug would be expected to disturb its functional activity, the disturbance being proportional to the degree to which the respiratory activity is affected. The action of the drug, hence the fall in respiratory activity, may be highly localised ; a large fall, therefore, in the respiration of the entire nervous system by biologically active concentrations of the drug might not be anticipated either *in vivo* or *in vitro*.

## Effects of Narcotics on the Respiration of Brain *in vitro*.

Narcotics at low concentrations have the power of inhibiting the respiration of brain tissue whether this be examined in the form of a mince [12] or in the form of thin intact tissue slices.[13]

The effects of seven alkyl barbiturates on the oxygen uptake of minced guineapig brain respiring in the presence of glucose are shown in Table I.[12]

TABLE I.

| Barbiturate (0·12 %). | Hypnotic Activity. | % Inhibition of Oxygen uptake of Minced Guineapig Brain in Presence of Glucose. |
|---|---|---|
|  | 0 | 6 |
|  | 0 | 4 |
|  | Very weak | 0 |
|  | + | 10 |
|  | ++ | 50 |
|  | ++ | 40 |
|  | ++ | 57 |

The relative hypnotic activities of the drugs are also shown, these being expressed by the symbols : O representing lack of activity, + a weak

[12] Quastel and Wheatley, *Proc. Roy. Soc. B*, 1932, **112**, 60.
[13] Jowett and Quastel, *Biochem. J.*, 1937, **31**, 565.

activity and ++ a relatively high hypnotic action. The following points of interest are worthy of note:

(1) Whereas *iso*propyl barbiturate is almost inert both as a hypnotic and as an inhibitor of oxidations, the *iso*propylallyl derivative is very active in both respects.

(2) The combination of *iso*propyl barbiturate with urethane leads to no appreciable increase in hypnotic or inhibitive powers.

(3) The introduction of a Br atom into allyl radicle of allyl*iso*propyl barbiturate does not affect appreciably the latter's hypnotic or inhibitive effects. The reduction, however, of the unsaturated linkage of the Br derivative to *iso*propyl-brompropyl barbiturate greatly reduces both hypnotic and inhibitive powers of the drug.

There is a rough parallelism in this series of barbiturates between hypnotic and inhibitive powers.

This parallelism is shown among narcotics of different chemical types. Thus chloral, which is a more powerful narcotic than paraldehyde, has also greater inhibitive effects on brain respiration *in vitro*. The same phenomenon is observable with hyoscine and atropine.

Using the more sensitive brain slice technique in which brain cortex tissue alone is used, it is possible to show [13, 14] that definite inhibitions of respiration take place in the presence of narcotics at concentrations which produce narcosis in animals. Results in Table II [14] make it clear that

TABLE II.—NARCOTISING CONCENTRATION AND EFFECTS ON THE RESPIRATIONS OF BRAIN CORTEX SLICES IN A GLUCOSE MEDIUM.

| Narcotic. | Animal. | Estimated Narcotic Dose g./kg. | Narcotising Concentration (M.). | % Inhibition of Isolated Brain Tissue Respiration due to Narcotising Concentration. |
|---|---|---|---|---|
| Ethyl urethane | Rat | 2 | 0·022 | 6 |
| Chloral hydrate | ,, | 0·22 | 0·0013 | 10 |
| Luminal | ,, | 0·2 | 0·00079 | 15 |
| Chloretone | ,, | 0·18 | 0·0010 | 20 |
| Evipan | Guineapig | 0·16 | 0·00062 | 17 |
| Avertin | Rat | 0·3 | 0·00106 | 31 |
| Chloretone | Guineapig | 0·18 | 0·0010 | 32 |

a variety of narcotics, at their narcotising concentrations, produces inhibitions of respirations varying from 6 % to 32 %. The results are not inconsistent with the view that the narcotics considered produce approximately equal inhibitions (of the order of 15 %) of the respiration of brain slices when present at concentrations which in the organism would produce an equal fairly deep narcosis. The data, as Jowett states, do not establish this view, but are sufficient to show that a definite inhibition of respiration is produced by concentrations of the order of those producing deep narcosis. The inhibitions recorded represent the effects of the narcotics on the respiration of the entire brain cortex of the animal; they may clearly be much greater at those parts of the nervous system where the narcotic becomes localised.

The action of narcotics on the combustion of all substrates attacked by the brain is not a general one. The oxidations of glucose, lactate and pyruvate are the most affected by narcotics, whilst the oxidations of succinate and *p*-phenylene diamine are undisturbed.[12, 13]

Results in Table III [12] show these effects, as well as indicating a sensitivity of glutamate oxidation to narcotics. These results were

[14] Jowett, *J. Physiol.*, 1938, **92**, 322.

obtained using minced guineapig brain. Data obtained by the more sensitive tissue slice technique are shown in Table IV [13] where the effects of luminal on rat brain cortex oxidations are recorded.

The high sensitivity of glucose oxidation in brain to the narcotics is a striking feature of narcotic action *in vitro* and, in view of the great importance of glucose oxidation in the functional activity of the central nervous system (see also [15]), this sensitivity must be a highly significant factor in any consideration of the mechanism of narcotic action.

TABLE III.—PERCENTAGE INHIBITION BY NARCOTICS (0·12 %) OF OXYGEN UPTAKES OF MINCED GUINEAPIG BRAIN PRODUCED BY VARIOUS SUBSTRATES.

| | Allyl*iso*propyl Barbiturate. | Luminal. | Chloretone. | Hyoscine. | Chloral-hydrate. |
|---|---|---|---|---|---|
| Glucose . . . | 73 | 94 | 93 | 79 | 66 |
| Na Lactate . . | 71 | 79 | 88 | 73 | 90 |
| Na Pyruvate . . | 67 | 85 | 84 | 71 | 90 |
| Na Succinate . | 2 | 0 | 0 | 0 | 0 |
| Na Glutamate . | 28 | 50 | 59 | 60 | 62 |
| *p*-Phenylenediamine | 0 | 0 | — | — | — |

TABLE IV.—EFFECTS OF LUMINAL (0·08 %) ON BRAIN RESPIRATION IN PRESENCE OF VARIOUS METABOLITES. (RAT BRAIN CORTEX SLICES.)

| Metabolite. | $Q_{O_2}$ without Narcotic. | $Q_{O_2}$ with Narcotic. | % Effect of Narcotic on $Q_{O_2}$. |
|---|---|---|---|
| Nil . . . . . | 2·9 | 2·75 | −5 |
| Glucose 0·01 M. . . | 12·2 | 5·5 | −55 |
| Na *d*. Lactate 0·02 M. . | 13·5 | 8·8 | −35 |
| Na Pyruvate 0·02 M. . | 11·1 | 8·1 | −27 |
| Na Glutamate 0·02 M. . | 8·0 | 6·8 | −15 |
| Na Succinate 0·02 M. . | 9·5 | 10·2 | +7 |

TABLE V.—EFFECT OF 0·033 % EVIPAN * ON RESPIRATION OF GUINEAPIG TISSUES IN PRESENCE OF GLUCOSE.

| Tissue. | Respiration ($Q_{O_2}$). | Respiration ($Q_{O_2}$) in Presence of Narcotic. | % Inhibition by Narcotic. |
|---|---|---|---|
| Brain . . | 14·2 | 9·5 | 33 |
| Spleen . . | 7·7 | 6·4 | 17 |
| Liver . . | 4·25 | 4·15 | 2 |
| Testis . . | 8·65 | 7·25 | 16 |
| Kidney . . | 15·2 | 15·95 | Nil. |

* Na *N*-methyl *cyclo* hexenyl methyl barbiturate.

The total respiration of tissues other than brain is also affected by narcotics, though not to the same degree. A result with evipan is shown in

[15] Quastel, Tennenbaum and Wheatley, *Biochem. J.*, 1936, **30**, 1668; Mann, Tennenbaum and Quastel, *ibid.*, 1938, **32**, 243.

Table V.[13] Examination [13] of the inhibitive action of narcotics on the respiration of a variety of tissues has shown that narcotics inhibit the oxidation of glucose, lactate and pyruvate in tissues such as liver, kidney or diaphragm to about the same extent as in brain. It is difficult, however, to state in quantitative terms the inhibitory action of, say, luminal on substrate oxidations by liver or kidney owing to the relatively rapid change of inhibition with time and the effect of the drug on the respiration of the tissue in absence of added substrate. It would seem, on the whole, that the effects of low concentrations of narcotics are confined as with brain to the inhibition of substances important in carbohydrate metabolism. With brain, however, in contrast to such tissues as liver and kidney, carbohydrate breakdown seems to be the dominant feature of metabolism and it is this fact which throws into prominence the specific inhibitory effects of narcotics in brain metabolism.

## Reversibility of Narcotic Action *in vitro*.

The effects of narcotics such as the barbiturates or chloretone, or hyoscine, on the respiration of brain cortex slices are not irreversible. This is shown [16] simply by washing the brain slices in a narcotic-free medium after their immersion for an hour at 37° in the narcotic solution. The low and steady oxygen uptake found in presence of the narcotic is raised immediately to a higher level which also remains steady. A typical result, with chloretone as the narcotic, is shown in Table VI.[16] Results showing the attainment of steady rates of respiration by brain cortex slices in presence of low concentrations of urethane, avertin or luminal are shown in Figs. 1, 2 and 3.[14] High concentrations of narcotics, however, produce irreversible effects.

TABLE VI.—GUINEAPIG BRAIN CORTEX SLICES IN PHOSPHATE-LOCKE-GLUCOSE MEDIUM.

| | $Q_{O_2}$ (Respiration). |
|---|---|
| 1*a*. In absence of narcotic | 15·5 |
| 1*b*. After washing and reimmersion in fresh medium | 14·2 |
| 2*a*. In presence of 0·002 M. chloretone | 5·8 |
| 2*b*. After washing and reimmersion in fresh narcotic free medium | 12·0 |

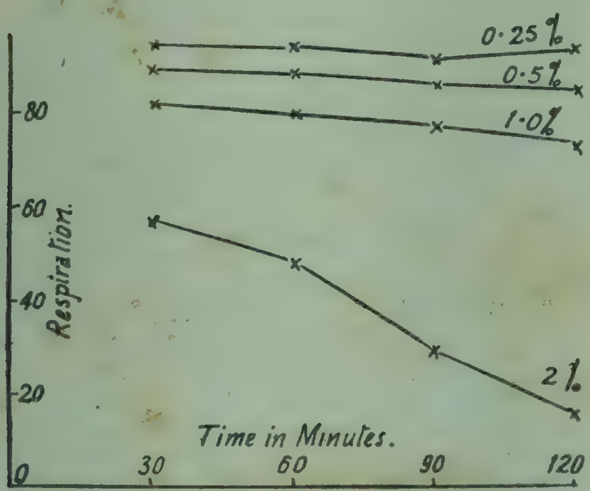



FIG. 1.—Effect of ethylurethane on the respiration of rat brain cortex in presence of glucose (Jowett).

Analysis of the data indicates that two effects of a narcotic on respiration of brain *in vitro* take place :

(1) Rapid attainment of an equilibrium between the narcotic and a constituent of the respiratory system. The inhibition of respiration is that to be expected from a simple mass action equation (Jowett,[14]), as observable with small concentrations of narcotic

[16] Quastel and Wheatley, *Biochem. J.*, 1934, 28, 1521.

producing inhibitions not greater than 40 %. This applies to narcotics such as urethane, chloral, chloretone, barbiturates, avertin (tribromethyl alcohol) and magnesium ions.

(2) Relatively slow development of irreversible changes, leading to increased inhibitions of respirations which cannot be restored to normal by removal of narcotic. This takes place with most narcotics but is only observable at relatively high concentrations with narcotics such as barbiturates or chloretone. It occurs, however, at low concentrations with ether [17] and ethyl alcohol.[14] Irreversibility of action also occurs with indole [16] which is also a powerful inhibitor of brain respiration. The reasons for these irreversible changes are obscure.

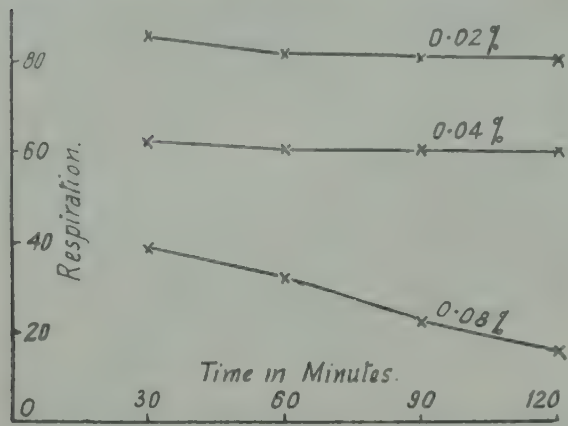



Fig. 2.—Effect of avertin on the respiration of rat brain cortex in presence of glucose (Jowett).

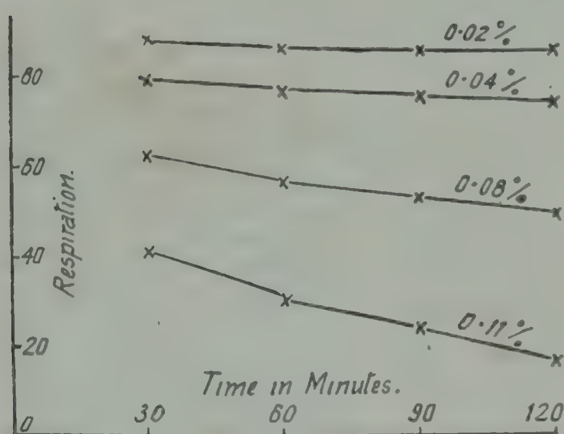



Fig. 3.—Effect of luminal on the respiration of rat brain cortex in presence of glucose (Jowett).

## Effects of Potassium Ions.

Another phenomenon bearing upon the inhibitory effects of small concentrations of narcotics may now be considered. The steady state of the diminished respiration of brain slices brought about by small concentrations of narcotics such as luminal or chloretone is found to be greatly dependent on the concentration of $K^+$ in the medium. With such high concentrations of $K^+$ as 0·0128 M.—double that normally present in serum—a steady inhibition is quickly attained by chloretone. At low concentrations of $K^+$, *e.g.* 0·002 M., however, the respiration is found to drop quickly in presence of narcotics to the level found with the higher $K^+$ concentrations,, to remain at this steady state for a short period and then to fall quickly so that eventually the inhibition found with low $K^+$ concentrations is very much greater than with the higher $K^+$ concentration (see Fig. 4 [13]). The concentration of $K^+$ normally present in serum is sufficient to stabilise the inhibition due to the narcotic. When the temperature of the experiment usually carried out at 39° is dropped to 29° the inhibitory effect of a narcotic such as chloretone no longer varies appreciably with the $K^+$ (see Fig. 5 [13]). A steady inhibition is found whether the $K^+$ is high or low. The increasing inhibition at 39° due to low concentrations of $K^+$ is possibly due to loss of $K^+$ from the nerve cell at the low external concentration or to loss of some other cell constituent

[17] Jowett and Quastel, *Biochem. J.*, 1937, 31, 1101.

concerned with cell respiration, due to irreversible changes in the cell which proceed more slowly when the temperature is lowered.

## Narcotics and Substrate Concentration.

If a narcotic enters into a mass action equilibrium with an enzyme with which the substrate oxidised by the brain is also combined, it would be expected that increase in substrate concentration would lead to a diminution in the inhibitive power of the narcotic. Results given in Table VII [18] show that this does not take place. An eight-fold increase in concentration of sodium pyruvate has no effect on the inhibition of oxygen uptake of rat brain cortex due to chloretone.

This shows that the narcotic must enter into equilibrium with some component of the respiratory system which is independent of the substrate burned.

Yet this conclusion is opposed to that obtained from the study of narcotic inhibition of brain dehydrogenations carried out under anærobic conditions.[18]

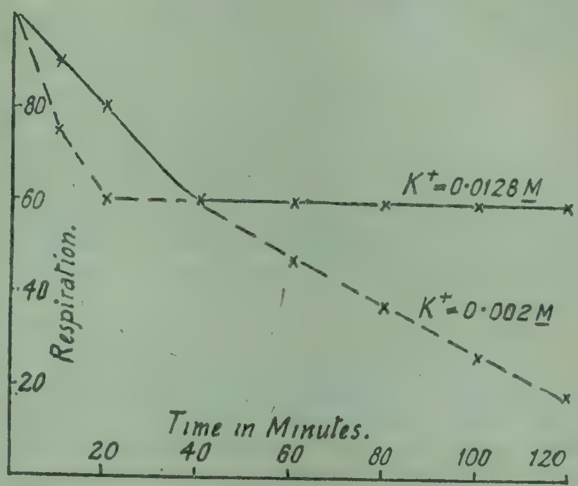



FIG. 4.—Effect of 0·033 % chloretone on the respiration of rat brain cortex in presence of glucose at 39° C. (Jowett and Quastel).
Temp. = 39° C.

## Narcotics and Dehydrogenases.

In a study [18] of the inhibitory effect of chloretone on lactic dehydrogenase of minced brain tissue, it was found that the narcotic and substrate (sodium lactate) competed, according to the mass action law, for the enzyme. The enzyme was studied in the usual way by determining the rates of reduction under anærobic conditions of a dyestuff, methylene blue, in presence of the tissue and mixtures of varying concentrations of narcotic and substrate.

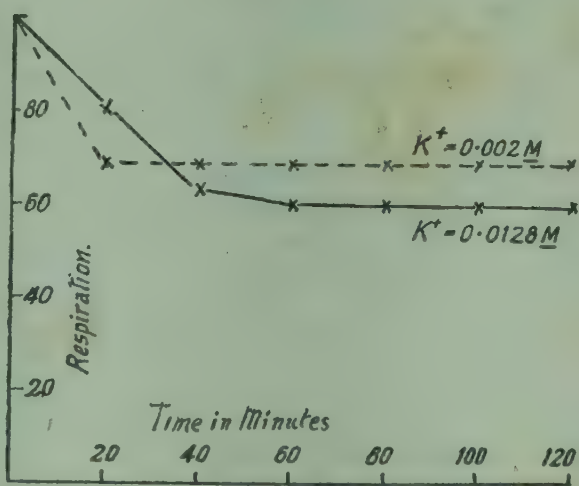



FIG. 5.—Effect of 0·033 % chloretone on the respiration of rat brain cortex in presence of glucose at 29° C. (Jowett and Quastel).
Temp. = 29° C.

It had long been known that narcotics inhibit dehydrogenases and it seemed a simple explanation of narcotic inhibition of brain respiration to suppose that the effect was due to inhibition of dehydrogenases affecting either glucose, or lactate or pyruvate. The results given in Table VII show that this cannot be the case. Yet the effect of the narcotic is not connected with the enzymes concerned with the activation of oxygen itself, as inhibitive concentrations of narcotics do not affect the oxidation of sodium succinate or *p*-phenylene diamine.

[18] Davies and Quastel, *Biochem. J.*, 1932, **26**, 1672.

TABLE VII.—EFFECT OF SUBSTRATE CONCENTRATION ON INHIBITIONS OF BRAIN CORTEX RESPIRATION ($Q_{O_2}$) BY NARCOTICS.

| Animal. | Substrate. | Narcotic. | % Inhibition of $Q_{O_2}$. |
|---|---|---|---|
| Rat | Na Pyruvate 0·01 M. | Chloretone 0·037 % | 37 |
| | ,, 0·08 M. | ,, | 37 |
| Guineapig | ,, 0·06 M. | Luminal 0·08 % | 27 |
| | ,, 0·01 M. | ,, | 28 |
| ,, | Na *d*. Lactate 0·06 M. | ,, | 39 |
| | ,, 0·01 M. | ,, | 31 |

A clue to the solution of this anomaly may be found in the fact that the low concentrations of narcotics which are highly inhibitory ærobically have but little inhibitory effect anærobically. Thus chloretone is ten times less effective in securing an inhibition of lactic acid oxidation in presence of brain anærobically than under ærobic conditions.

A possible explanation is that the narcotic, at the low concentrations effective under ærobic conditions, exerts its main effect on a part of the respiratory system which is inert or functionless under anærobic conditions. That such a part of the respiratory system has the properties of an enzyme structure is evident from the fact that it is affected by surface active narcotics of so many different chemical types. It must, however, be a tissue component with a much higher affinity for narcotics than the most sensitive enzymes—the dehydrogenases—with which we are so far familiar.

If this view is true the inhibitions of respiration of intact brain tissue obtained by low concentrations of narcotics under ærobic conditions are not due to competition of the narcotics with the substrates (*e.g.*, lactic or pyruvic acid) for their dehydrogenases but to the affinity of the narcotics to a special component playing a highly important part in the chain of reactions composing the complete ærobic respiratory process of the cell.

## Narcotics and Glycolysis.

Confirmation of the fact that narcotics at low concentrations do not affect the known dehydrogenases comes from the entire absence of any inhibition by a narcotic such as chloretone on anærobic glycolysis by brain cortex (see Table VIII [19]). The mechanism of anærobic breakdown of glucose in brain is not yet clear but if the reactions involved are similar to those taking part in the anærobic breakdown of glucose in muscle or yeast, an interplay of dehydrogenase systems will be involved. The results show that if a narcotic-sensitive dehydrogenase system plays an important role in the ærobic breakdown of glucose it is either absent from, or is without influence in, the reactions involved in the anærobic breakdown of glucose by brain.

TABLE VIII.—EFFECT OF CHLORETONE ON ANÆROBIC GLYCOLYSIS ($Q_M^{N_2}$) OF RAT BRAIN CORTEX.

| Conc. of Narcotic. | $Q_M^{N_2}$. | | |
|---|---|---|---|
| Nil | 15·2 | 19·8 | 16·5 |
| 0·033 % | 15·1 | — | — |
| 0·05 % | — | 17·8 | 16·4 |

## Narcotics and an Intermediate Respiratory Component.

An investigation [19] of respiratory systems made up from isolated tissue components, *e.g.*, muscle dehydrogenases, and preparations of cozymase,

[19] Michaelis and Quastel, *Biochem. J.*, 1941, **35**, 518.

flavoprotein, and cytochrome, oxidase, and studied under both ærobic and anærobic conditions, has shown that the effect of narcotics at low concentrations is restricted to a tissue component, which is possibly a flavoprotein. This is illustrated in Fig. 6 which shows the narcotic sensitive and insensitive regions of a narcotic sensitive respiratory system.[19] It is impossible, however, with our present incomplete knowledge of the components of respiratory systems, to state definitely which component is specifically affected at the low concentrations of narcotics which inhibit brain respiration. It is, however, a step forward to realise that a special component of the respiratory system is highly narcotic sensitive, and that the effects of narcotics at biologically important con-

Narcotic Sensitive Respiratory System

Substrate—Dehydrogenase—Cozymase—Flavoprotein—Cytochrome—Cytochrome Oxidase—Oxygen

Narcotic Insensitive Region — Narcotic Sensitive Region — Narcotic Insensitive Region

FIG. 6.

centrations are not to be attributed, as has been supposed in the past, to a general inhibition of dehydrogenases by non-specific surface adsorption of the narcotics.

## Benzedrine and Amine Oxidase of Brain.

It is well known that benzedrine administration has a considerable value in cases of narcolepsy and in conditions where a stimulation of the central nervous system is required.

The addition of benzedrine at low concentrations to brain cortex respiring in a glucose medium neither increases nor decreases the respiration, nor will it neutralise the diminution of respiration due to the presence of a narcotic.

It is possible, however, that the relation of benzedrine to amine oxidation in brain may be connected with its clinical effects.

It has been known for some time [20] that the presence of amines such as tyramine or *iso*amylamine brings about a marked diminution (increasing with time) in the respiration of brain examined *in vitro*. The addition of benzedrine, however, to brain cortex respiring in the presence of tyramine and other inhibitive amines has been found to neutralise, or partly neutralise, the inhibition.[21] A typical result is shown in Table IX.[21]

TABLE IX.—EFFECTS OF BENZEDRINE ON RAT BRAIN CORTEX RESPIRATION IN PRESENCE OF TYRAMINE IN A GLUCOSE MEDIUM.

| Tyramine M. Conc. | Benzedrine M. Conc. | $Q_{O_2}$ | | |
|---|---|---|---|---|
| | | 1st Hour. | 2nd Hour. | 3rd Hour. |
| 0 | 0 | 11·4 | 10·1 | 9·0 |
| $1·9 \times 10^{-3}$ | 0 | 9·6 | 5·3 | 3·1 |
| $1·9 \times 10^{-3}$ | $1·4 \times 10^{-4}$ | 10·1 | 8·5 | 6·3 |

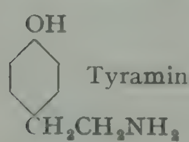
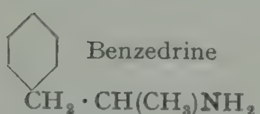
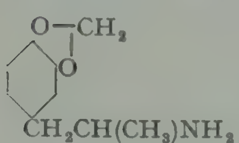
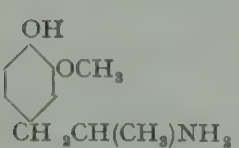
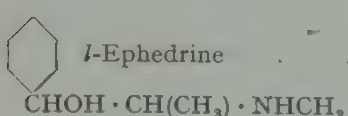
[20] Quastel and Wheatley, *Biochem. J.*, 1933, **27**, 1609.
[21] Mann and Quastel, *ibid.*, 1940, **34**, 414.

The fall in brain respiration due to the presence of tyramine, etc., is not due wholly to the amine itself but to a product of oxidation of the amine, *i.e.*, the corresponding aldehyde. Benzedrine owes its stimulating action in brain respiration in presence of inhibitive amines to its ability to compete reversibly with amines for the amine oxidase of brain, thereby reducing the rate of formation of the inhibitory aldehyde.

Competition for amine oxidase takes place between various amines. Pugh and Quastel [22] found that competition takes place between the following amines: tyramine, *iso*amylamine, β-indolethylamine and β-phenylethylamine. Blaschko *et al.*[23] showed that competition occurs between tyramine, adrenaline, *iso*amylamine and β-indolethylamine. They also showed that *l*-ephedrine, triethylamine, tri*iso*amylamine, hordenine—amines which are either slowly or not attacked by amine oxidase—have an affinity for the enzymes and inhibit the oxidation of amines which are attacked by the oxidase. Benzedrine is either very feebly or not at all attacked by amine oxidase. It has nevertheless a high affinity for this enzyme and is a powerful inhibitor of amine oxidation.

It has been proved that amines combine with amine oxidase according to the mass action law and that competition between the amines takes place in strictly reversible manner. Taking the dissociation constant of the tyramine-amine oxidase complex to be 0·00178,[23] the dissociation constants for benzedrine—and other amine-amine oxidase complexes have been worked out. These are shown in Table X.[21]

TABLE X.

| Amine. | Dissociation Constant of Amine Oxidase Complex with Amine. |
|---|---|
|  Tyramine | 0·00178 |
|  Benzedrine | 0·0004 |
|  | 0·0004 |
|  | 0·0050 |
|  *l*-Ephedrine | 0·0057 |

The effects of benzedrine *in vitro*, in partially neutralising the inhibition of glucose oxidation by brain due to the presence of tyramine and other amines, take place at concentrations which are not markedly greater than those which have pharmacological effects *in vivo*. Moreover, since competition between benzedrine and other amines takes place at amine oxidase according to the laws of mass action, it follows that the influence of benzedrine may be exerted in the body at much lower concentrations than have been used in *in vitro* investigations. It seems not unreasonable to suggest that the action of benzedrine *in vivo* is linked with its ability to compete with amines which give rise by oxidation to toxic substances; the lower the concentrations of such amines the lower the quantity of benzedrine which will be required to compete successfully with them.

[22] Pugh and Quastel, *Biochem. J.*, 1937, **31**, 2306.
[23] Blaschko, Richter and Schlossman, *ibid.*, 1937, **31**, 2187.

Ephedrine is known clinically to be less powerful than benzedrine as a stimulant of the central nervous system and it has been shown that such a difference between their effects on brain respiration occurs also *in vitro*. According to Gunn *et al.*[24] 3 : 4 methylene dioxyphenyl*iso*-propylamine is a somewhat more powerful stimulant of the central nervous system than benzedrine and it has at least the same activity as benzedrine in neutralising the fall of brain respiration in a glucose medium due to tyramine. Again, according to Gunn *et al.*[24] 3-methoxy-4-hydroxyphenyl-*iso*propylamine is a less powerful stimulant of the nervous system than benzedrine. The result is consistent with the relatively poor activity of the former amine in neutralising the inhibitory action of tyramine on brain oxidation.[21] Such results are suggestive of a possible connection between amine metabolism in the brain and the development of the clinical condition known to be relieved by benzedrine administration.

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[24] Gunn, Gurd and Sachs, *J. Physiol.*, 1939, **95**, 485.

## *GENERAL DISCUSSION*

**Dr. H. McIlwain** (*Sheffield*) (*communicated*) : Studies of the action of narcotics on bacteria [25] have given results which may be relevant to their action in the systems examined by Dr. Quastel and referred to also by Dr. Hurst and Dr. Ing. It was concluded that narcotics (ether, alcohol, chloroform, ethylcarbonate, novocaine) acted by reversibly denaturing enzymes of dehydrogenase systems, the critical evidence being antagonism of their action, by increased hydrostatic pressure. The antagonism was explained through the increase in volume accompanying denaturation, and was not found with bacterial inhibitors known to act differently, *e.g.* with sulphanilamide. The absence, found in several cases by Dr. Quastel, of antagonism to narcotics by substrate molecules is then understandable, but differences in susceptibility of proteins to reversible denaturation, and their varying importance to the cell, permits the specificity of action emphasised by him.

[25] Johnson, Brown and Marsland, *Science*, 1942, **95**, 200 ; *J. Cell. comp. Physiol.*, 1942, **20**, 269 ; Johnson, Eyring and Williams, *ibid.*, 1942, **20**, 247.

# RELATIONS BETWEEN *IN VIVO* AND *IN VITRO* ACTIONS OF CHEMOTHERAPEUTIC AGENTS.

BY HENRY McILWAIN.

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Study of the relation between the action of chemotherapeutic agents on the course of microbial infections, and upon the parasites alone, is a means of analysing the complex system presented in practice by the host, parasite, and drug. As activity or inactivity of the agents is conditioned much more by the nature of the parasite than by that of the host, they have been expected to exhibit some action on the parasite outside the host. Such action has not always been found and, in consequence, various types of interactions between drug and host have been postulated. Most of

these are discredited, but of them, the conversion of the drug to more active forms undoubtedly occurs *in vivo* in certain cases. Most important among other causes of apparent *in vitro* inactivity of therapeutically active agents are inadequacies in the technique of *in vitro* experiments. Notable instances are afforded by the poor cultural conditions under which pathogenic trypanosomes and amoebae were first studied, which prevented observation of the effect of agents whose action was not manifest in a few hours; for the parasites soon died.[1, 2] The present account emphasises other instances which show that, for chemotherapeutic studies, culture media must not merely be adequate for growth, but must simulate conditions in the host in respect to their concentrations of specific substances, commonly occurring in animals and media, which antagonise drug action.

## Actions of the Host on the Drug; Types of *in vitro* Activity.

Before considering those interactions which are susceptible to *in vitro* study, it is necessary to emphasise some basic ones which are not. For the purpose of quantitatively comparing the action of a compound *in vitro* and *in vivo*, it is at present necessary to administer it to animals and find what concentrations can be attained in relevant sites without damage to the animal. The levels are demonstrably conditioned by a combination of absorption, metabolism, and excretion. Some of these processes can be studied *in vitro*—*e.g.*, the acetylation and inactivation of sulphanilamide by the liver, or the conversion of Prontosil to the active sulphanilamide by various tissues.[3]

The type of *in vitro* action which is associated with activity of an agent *in vivo* has often been assumed to be the killing of the parasite. This, however, occurs only in some cases. Acriflavine was found to be primarily bacteriostatic *in vitro*, and it was emphasised that such an action, combined with the normal behaviour of the host towards non-invasive bacteria, was adequate for disinfection *in vivo*.[4] This has been reiterated with respect to the sulphonamides. From the many observed and postulated activities of the sulphonamides, which have included the stimulation of defences of the host and various actions on the parasites, their bacteriostatic action has been accepted as adequate to explain *in vivo* activity.[3, 5] Although it is not necessarily a general conclusion that inhibition of growth is the basis of bacterial chemotherapy, no case which has been fully investigated is inconsistent with the suggestion. It has been successfully used as a working hypothesis; [6] other actions are examined below.

## Natural Occurrence of Compounds Antagonistic to Chemotherapy.

An early instance of a specific participation of the host in chemotherapy was encountered in the activation of certain arsenicals by their conversion to arsine oxides. Conversion to a more active compound was also observed with Prontosil, and when some workers found sulphanilamide itself to be inactive *in vitro*, this also was referred to interactions between the drug and the host. In this case, however, reports of *in vitro* studies were not consistent, some observers finding the compound highly active. Many of these discrepancies were explained in a series of papers, now very familiar, in which they were shown to be largely due to different media; material was extracted from various organic sources which prevented the

[1] Yorke and Murgatroyd, *Ann. Trop. Med. Parasitol.*, 1930, **24**, 449.
[2] Dobell and Laidlaw, *Parasitology*, 1926, **18**, 206.
[3] Marshall, Jr., *Physiol. Rev.*, 1939, **19**, 240; *Ann. Rev. Physiol.*, 1941, **3**, 643.
[4] Browning and Gulbransen, *J. Hyg.*, 1919-20, **18**, 33.
[5] Fleming, *Proc. Roy. Soc. Med.*, 1940, **33**, 487.
[6] McIlwain and Hawking, *Lancet*, 1943, **1**, 449.

bacteriostatic action of the drug, and the main antagonist was shown to have the properties of *p*-aminobenzoic acid. Thus the greater *in vivo* activity of sulphanilamide was referred to a phenomenon which was more familiar in chemotherapy as having the opposite effect. The more usual action is illustrated by the *in vivo* inactivity of mercuric compounds, which was shown due to their combining with a particular protein of serum,[7] or by some phenolic compounds, which were sufficiently active in broth to give expectations of *in vivo* activity, but again were antagonised by serum though no evident combination occurred.[8] On the other hand, the activity of acriflavine was greater in serum than in broth, though it was lowered by pus ; here again, specific materials have been implicated.[9] Clearly, therefore, there are different types of antagonistic compounds, of varying natural occurrence, which interfere in different ways with the actions of various chemotherapeutic agents and whose activities must be assessed before the activity of a given concentration of an agent *in vitro* can be compared with that *in vivo*.

## Nature of Drug Antagonists.

The nutritional relationship between host and parasite is a fundamental one, and was early emphasised in relation to chemotherapy. Analogies were indicated between the action of drugs and conditions of poor nutrition,[10] and related to such a conception is the regarding of drug-antagonists as nutrients or as stimulating the proliferation of the parasite.[11] With growth of biochemical knowledge, the importance to living organisms of compounds other than those assimilated as foodstuffs has been made evident, and Voegtlin [12] found one such metabolite to be a drug-antagonist : phenylarsine oxides formed dissociable compounds with glutathione and other thiol compounds which annulled their trypanocidal action *in vivo* and *in vitro*. Moreover, he suggested such combination as a basis not only for antagonism of the arsenicals, but also of their action upon the parasite, for thiol compounds were known to be of critical importance in living processes. The actions of many inhibitors on metabolic processes had been investigated at the time when sulphanilamide-antagonism was being studied, and Woods [13] applied the knowledge that enzymes can be inhibited by compounds structurally related to their substrates, in suggesting *p*-aminobenzoic acid as both an essential metabolite [14] to the bacteria, and as the compound responsible for antisulphanilamide activity in his preparations.

The relationship between sulphanilamide and *p*-aminobenzoate proved an extremely specific one, though other compounds, notably purines and methionine, antagonised the action of sulphanilamide in other ways.[15] Consequently the demonstration that the interactions occurred *in vivo* [16] as well as *in vitro* was valuable evidence that the anti-streptococcal action of sulphanilamide *in vivo* was indeed the same as that *in vitro*. The *in vivo* antagonism of pentavalent arsenicals by thiol derivatives had previously been used as evidence of the similarity of their action to that of the arsine oxides and thus to confirm the suggestion that the pentavalent compounds functioned only after their reduction in the host.[12]

Results suggestive of the natural function of drug-antagonists have

[7] Smith, Czarnetzky and Mudd, *Amer. J. Med. Sci.*, 1936, **192**, 790.

[8] Bechhold and Ehrlich, *Hoppe-Seyl. Z.*, 1906, **47**, 173.

[9] McIlwain, *Biochem. J.*, 1941, **35**, 1311.

[10] Braun and Schaeffer, *Z. Hyg. InfektKr.*, 1919, **89**, 339 ; Stearn, E. A. and Stearn, E. W., *J. Bact.*, 1924, **9**, 491.

[11] Lockwood, *J. Immunol.*, 1938, **35**, 155 ; Green, *Brit. J. exp. Path.*, 1940 **21**, 74.

[12] Voegtlin, *Physiol. Rev.*, 1925, **5**, 63.

[13] Woods, *Brit. J. exp. Path.*, 1940, **21**, 74.

[14] Fildes, *ibid.*, 1940, **21**, 67.

[15] Kohn and Harris, *J. Pharmacol.*, 1943, **77**, 1.

[16] Selbie, *Brit. J. exp. Path.*, 1940, **21**, 90 ; Martin and Fisher, *J. biol. Chem.*, 1942, **144**, 287.

also been obtained by designing inhibitors to interfere with known bacterial metabolites,[17] assuming them to be related to enzyme systems which could be regarded as potential drug-receptors.[18] Many compounds structurally related to such metabolites were found inhibitory, and their actions in most cases were antagonised by the metabolites used as models. Consideration of the naturally-occurring concentrations of their antagonists showed that some would not be expected to be active *in vivo*. Pantoyltaurine,[18] however, inhibited streptococci *in vitro* in the presence of the concentration of pantothenate (its main antagonist) in rat blood, to degrees comparable with those associated with therapeutic activity in sulphanilamide,[19] when present in concentrations which could be attained in rats. Action *in vivo* was hence anticipated,[20] and found ; [6] here also the essential similarity of the action *in vivo* and *in vitro* was shown by preventing the *in vivo* action with more pantothenate.

## Varying Occurrence and Metabolism of Drug Antagonists.

The concentration of an inhibitor necessary for bacteriostasis *in vitro* is in some cases roughly proportional to the coincident concentration of antagonists ; [13, 17, 20] other relationships have been found in other cases,[9, 17] but all emphasise that the naturally occurring concentrations of drug antagonists, and their alteration by metabolism of the host, can be as important to the action of a drug *in vivo* as can the concentration and metabolism of the drug itself. Thus, sulphonamide antagonists presented some puzzling phenomena. The quantities of *p*-aminobenzoate needed for preventing the antistreptococcal activity of sulphanilamide when the two compounds were fed or injected together,[16] were about 500 times those which were effective *in vitro*.[19] Again, the quantities of sulphanilamide needed for antistreptococcal action in untreated tissue fluids or simple extracts were only a fraction of those needed in the presence of other extracts (*e.g.*, some bacteriological broths) or autolysates (*e.g.*, pus). These results were explicable by the action of the host on one of the drug antagonists, either in conjugating *p*-aminobenzoate when administered or in liberating it from combined forms on autolysis.[19] Normal human blood and urine, indeed, were found to contain quantities of combined *p*-aminobenzoate in great excess of that needed to antagonise the anti-streptococcal action of the highest sulphonamide levels attainable *in vivo*. This indicates the vital importance of some normal mechanism, possibly acetylation, to all sulphonamide therapy. Of practical importance is the finding that sulphonamide activity *in vivo* is antagonised by certain local anaesthetics.[21] These contained *p*-aminobenzoate and their activity had first been demonstrated *in vitro* ; [13] the *in vivo* activity was not great, presumably for the reasons discussed in relation to *p*-aminobenzoate itself.

Allied to these results is the finding that azochloramid potentiates the sulphonamides ; [22] the action extends to *in vivo* conditions and has been explained as a destruction of sulphonamide antagonists,[23] including those synthesised by the parasite itself. The finding should be of more general application, and microbial destruction and synthesis of drug antagonists is known, which can afford a basis for both greater and lesser susceptibilities of mixed infections, to a given drug.

Considering again conditions in the host, notable differences are known

[17] McIlwain, *Brit. J. exp. Path.*, 1940, **21**, 136 ; *ibid.*, 1941, **22**, 148 ; Fildes, *ibid.*, 1941, **22**, 293.

[18] McIlwain, *Lancet*, 1942, **1**, 412.

[19] McIlwain, *Brit. J. exp. Path.*, 1942, **23**, 265.

[20] McIlwain, *ibid.*, 1942, **23**, 95.

[21] de Waal, Kanaar and McNaughtan, *Lancet*, 1942, **2**, 724.

[22] Neter, *J. Pharmacol.*, 1942, **74**, 52 ; *J. Bact.*, 1942, **44**, 261.

[23] Schmelkes and Wyss, *Proc. Soc. exp. Biol., N.Y.*, 1942, **49**, 263.

to exist in the concentrations of metabolites, including drug antagonists, in different animal species.[24] The values for pantothenate (antagonising pantoyltaurine) in the same tissues of different animals (mouse, rat, hog and cow) could vary over a three-fold range, and during the life of a given species showed variations up to four-fold, with maxima in different tissues and different animals occurring at different times. Concentrations of nicotinic acid derivatives (relevant to the action of certain sulphonamides)[25] were found to be more constant, but of riboflavin (partly antagonising acriflavine[9]) in different species showed a variation greater than ten-fold, the maximum again being different in time in different species and organs. A point of special importance in relation to animal testing of chemotherapeutic agents is the finding of a general tendency to inverse relationship between the size of animal and the level of essential metabolites, including drug-antagonists, in their tissues.[24] This would tend to cause lesser action or inactivity in, *e.g.*, mice, with agents which might be active in the larger animals with which chemotherapy is ultimately most concerned. Such results have been obtained,[2, 6, 26] and sometimes referred to varying metabolism of the drug; the present considerations offer alternative explanations. The general rough agreement in chemotherapeutic effects in hosts of different species is parallelled by the finding that differences in metabolite levels in different species was not commonly greater than ten-fold. Complete differences in metabolism of compounds related to drug antagonists are, however, known to exist in different animal species, *e.g.*, the unusual reaction of fowls to substituted benzoic acids, or of man to phenylacetic acids.[27]

## Actions of Chemotherapeuticals on Parasites *in vitro*, other than those on Growth.

Many attempts have been made to correlate the actions of chemotherapeutic agents with properties other than their effects on growth; those relating to purely chemical and physical properties will be discussed by other contributors.

Metabolic methods have been used to measure growth of organisms, and so indirectly to show parastiostatic or parasiticidal action,[28, 29] but this section is intended to consider the more direct action of inhibitors on metabolic processes. Sulphanilamide appears to have no action on respiration[3] at concentrations at which it inhibits growth and is active therapeutically, though non-specific effects occur with higher concentrations. Sulphapyridine affects the oxidation of three-carbon compounds, though this action, unlike the major effects of the drug, is not antagonised by *p*-aminobenzoic acid.[25] Many simple antibacterial agents (mercuric salts, phenols, iodine) inhibit respiration of *Bact. coli*, as also does rivanol, and such inhibition has been suggested as a measure of germicidal powers; its connection with bacteriostasis is not, however, very close, and the instance of sulphanilamide would preclude its general application to chemotherapy. Metabolic methods have also been used in assessing the action of antimalarials on the protozoa;[30] in this case the experiments were performed

[24] Williams and others, *Studies on the Vitamin Content of Tissues*, I and II; Univ. Texas Publ. 4137 and 4237, 1941 and 1942. The values quoted were found by microbiological assay after standardised processes of extraction.

[25] Dorfmann and Koser, *J. inf. Dis.*, 1942, **71**, 242.

[26] Schlossberger, *Z. Hyg. InfektKr.*, 1928, **108**, 627.

[27] Harrow and Sherwin, *Textbook of Biochemistry*, Saunders, 1935.

[28] Greig and Hoogerheide, *J. Bact.*, 1941, **41**, 557; Hirsch, *Studien uber die mikrobiologischen Grundlagen der Sulphanilamid-Therapie*, 1942, Kenan Basimevi, Istanbul.

[29] Bronfenbrenner, Hershey, and Doubley, *J. Bact.*, 1939, **37**, 583.

[30] Fulton and Christophers, *Ann. Trop. Med. Parasitol.*, 1938, **32**, 77; Wendel, *J. biol. Chem.*, 1943, **148**, 21.

in body fluids with little modification, but much of the work with bacteria has used media whose content of drug antagonists may be very different from that *in vivo*. Many examples of the action of chemotherapeutic agents on particular enzymes can be given,[31] and in some cases parallelism has been reported between actions on enzymes, and therapeutic activity; such a relation was found between trypanocidal and anti-fumarase action, though it is not necessarily considered of functional importance.[32] Indeed, the actions which have been most fully investigated, as those of the sulphonamides on carbonic anhydrase or peroxidase, have been shown to be unrelated to the action on the parasite. Evidence is not available concerning others, such as the high activities of quinine or atoxyl on lipases. Lack of correlation of antibacterial action with both overall respiration, and with effects on such enzyme systems as have been studied, emphasises the biochemical specificity of chemotherapeutic agents. The present ignorance of their detailed mode of action is parallelled by ignorance of that of many other compounds which exert critical effects on living organisms.

## *In vitro* Investigations of Actions of Drugs on Tissues and Processes of the Host.

It follows from the knowledge that some chemotherapeutic agents are primarily bacteriostatic, that natural defences of the host are of critical importance in their use; but not all these processes are susceptible to *in vitro* study. Special attention has been given to the action of acriflavine [4] and other acridine derivatives [33] on phagocytosis, and the motility of leucocytes. Many observers, in studying the effects of sulphonamides on phagocytosis, had been expecting the drugs to increase such activity, but an assessing of various reports [3] suggests that the compounds have little if any action upon the process other than that referable to the action of the drugs on the bacteria. This is emphasised by observations with sulphapyridine in infections of mice in which the reactions of the host differed with different parasites: the response to streptococci and drug being phagocytosis, but to pneumococci with the same drug, the development of immune bodies.[8] Antibodies have been observed *in vitro* to convert a bacteriostatic effect of sulphanilamide to a bactericidal one.[3] Again, in treatment of trypanosomiasis, immunity follows chemotherapy but its grade depends upon the strain of parasite and not upon the chemotherapeutic agent.[34] In view of these widely different cases of varying reactions of the host to inactivation of organisms by the same drug, special evidence would be needed to support hypotheses of a drug exerting a major direct effect upon the host's defences.

*In vitro* studies of the effects of chemotherapeuticals upon tissues of the host have also been made by tissue-culture and manometric methods. The value of *in vitro* observation of simultaneously growing tissues and parasites was emphasised in 1916,[35] but many reports have concerned only the brief exposing of tissues and bacteria to the agent, not necessarily in body fluids or solutions equivalent in content of antagonistic compounds.[35, 36] The sulphonamides were found to have extremely little action on chick embryo tissues in a critical study in which other differences between conditions in culture and in the animals are discussed: the

[31] Clark, *Heffter's Handbuch der Experimentellen Pharmakologie*, 1937, suppl. vol. 4.
[32] Quastel, *Biochem. J.*, 1931, **25**, 1121.
[33] Albert, Francis, Garrod, and Linnell, *Brit. J. exp. Path.*, 1938, **19**, 41.
[34] Browning, *System of Bacteriology*, Med. Res. Council, Lond., 1931, **6**, 501.
[35] Lambert, *J. exp. Med.*, 1916, **24**, 683.
[36] Salle, McOmie, Shechmeister, and Foord, *J. Bact.*, 1939, **37**, 639.

absence of circulatory and detoxicating mechanisms, and varying resistances of tissues from different animals.[37]

Manometric methods have also tended to ignore the importance of natural antagonists and of bacteriostatic effects in comparing the actions of compounds upon tissue and parasite. Decrease in respiration or glycolysis is commonly measured, and the relation of such a property to the actions of the agents in chemotherapy can only be established empirically, and can vary with the agent and tissue, which limits the value of the methods for even comparative purposes. Nevertheless, their potential value in selecting compounds suitable for use as antiseptics in brain operations has been shown.[38] In this case the problem itself defined the tissue to be used, but for more general purposes the choice appears to be arbitrary. Variations in concentration of drug antagonists are extremely large in different tissues, those of human origin varying twenty- to twenty-five-fold in their riboflavin and pantothenate contents, and six-fold in that of nicotinic acid derivatives.[24]

## Special Effects of Chemotherapeuticals on Parasites, Exhibited *in vivo* and *in vitro*.

In addition to the specific interaction between chemotherapeutic agents and their natural antagonists, referred to above, two related [18] types of behaviour, first observed *in vivo*, have been reproduced *in vitro*. These are drug resistance and chemotherapeutic interference.

Evidence was given for drug-resistance in trypanosomiasis, in which it was first observed, being conditioned largely by the host; but such a conclusion has been discredited.[39] Trypanosomes made resistant in one host have been found resistant in others, and *in vitro*; resistant organisms prepared *in vitro* can be resistant *in vivo*. Here the success or failure of chemotherapy is conditioned by an experimentally induced change in the parasite alone. Evidence has not been encountered for a drug alone causing a comparable change in a host, which persisted after the drug had been excreted; though a drug in the presence of the parasite *in vivo* can result in a lasting change in the natural defences of the host. Reasons other than drug-resistance in the parasite can, of course, condition failures in treatment, but after taking such factors into account a considerable number of such failures have been directly referred to properties of the parasite which can be demonstrated *in vitro*.[39, 40] Results with sulphonamides present closely analogous features and make the conclusions reached with respect to non-bacterial infections more general. Strains produced by use of one sulphonamide, *in vivo* or *in vitro*, are commonly resistant to all drugs of the group, though some specificity has recently been found in respect to *Shigella* strains.[41] Accurate measurement of the resistance of *Bact. coli* strains to different sulphonamides has also shown their relative resistance to sulphanilamide and sulphathiazole to vary with the media in which they were tested.[42] This is readily understandable in terms of drug antagonists and could conceivably give a basis for small differences in resistance of a given organism in different hosts on account of their varying blood constitutents.

Chemotherapeutic interference was first observed between acriflavine and parafuchsine, with trypanosomes in mice.[43] It has since been

[37] Jacoby, Medawar, and Willmer, *Brit. Med. J.*, 1941, **2**, 149.
[38] Manifold, *Brit. J. exp. Path.*, 1941, **22**, 111.
[39] Yorke and Hawking, *Ann. Trop. Med. Parasitol.*, 1932, **26**, 215; Yorke and Murgatroyd, *Trans. Roy. Soc. Trop. Med. Hyg.*, 1935, **28**, 435.
[40] Petro, *Lancet*, 1943, **1**, 35.
[41] Cooper and Keller, *Proc. Soc. exp. Biol., N.Y.*, 1942, **51**, 238.
[42] Harris and Kohn, *J. Immunol.*, 1943, **46**, 189.
[43] Browning and Gulbransen, *J. Path. Bact.*, 1922, **25**, 395.

observed in other systems, including that of arsenoxides and various dyestuffs with trypanosomes, when parallel effects were found *in vitro*.[44] Interfering effects on the metabolism of trypanosomes and yeast with acriflavine and parafuchsine have also been reported.[45] There is thus little doubt that this phenomenon also is independent of the host. The action of acriflavine on *Bact. coli in vitro* is reduced by various compounds, including phenosafranine, methylene blue and riboflavin, and interpretations of the phenomena have been given which link it with known effects of inhibitors on metabolic processes. Comparable phenomena are also known to occur in the much simpler system presented by enzymes *in vitro*.[46]

## Conclusion.

It is not intended in this survey to disparage the use of animal testing in chemotherapy, but to emphasise (1) that such testing is not necessarily much nearer to the conditions under which the drug will ultimately be used, than are properly chosen *in vitro* conditions ; and (2) that suggestions that chemotherapeuticals exert a major direct effect upon the host, or that the host in their presence acts upon the parasite in a manner different from that exhibited towards any micro-organism, non-invasive by nature or inhibition, must be carefully re-assessed. *In vitro* testing is known not to reproduce all the conditions of the normal environment of the parasite, but with present knowledge it is less likely to introduce new and unknown factors than is testing in another host. By trial and error, experimental hosts of roughly equivalent behaviour to man with respect to specified drugs may be found, but it would be extremely unlikely for such a relation to apply to a wide range of compounds active against a particular parasite, as such agents can have completely different antagonists whose natural occurrences and metabolism vary independently. The testing of antimalarials in canaries, though giving results comparable to those of therapeutic practice with some agents, fails with others.[47] That the agents giving concordant results are those of major importance is to be expected, for the majority were either used in choosing the conditions for the test or devised by it. In order to be discovered by use of the test, new agents must satisfy the stringent condition of activity in two unrelated hosts. Clearly, *in vivo* and *in vitro* examinations of agents are complementary ; thus the use of *in vitro* testing in selecting compounds suitable for further examination is valuable, though *in vitro* studies have an analytical importance beyond this. Much still remains to be understood of the manner in which chemotherapeutic agents inhibit parasites, or otherwise render them susceptible to body defences ; comparison of *in vivo* and *in vitro* effects has as yet been only semi-quantitative and its further study appears a promising approach to the problem.

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[44] von Jancso, N. and H., *Z. ImmunForsch*, 1936, **88**, 275.
[45] Scheff and Hasskó, *Zbl. Bakt.*, I, 1936, **136**, 420 ; Wright and Hirschfelder, *J. Pharmacol.*, 1930, **39**, 39.
[46] Quastel and Yates, *Enzymologia*, 1936, **1**, 60.
[47] Bishop, *Parasitology*, 1942, **34**, 1.

## GENERAL DISCUSSION

**Dr. H. Hurst** (*Cambridge*) (*communicated*) : The change in drug activity with variation in the carrier medium suggests that carrier activity may play a part by inducing a corresponding change in the functional susceptibility of the test organisms. Since the interpretation of toxicological data *in vivo* must ultimately depend on comparison with *in vitro* tests, a study of the mechanism and limits of carrier activity in physiological media may well fill a gap in our present knowledge of the factors which influence chemotherapeutic activity *in vivo*.

**Dr. F. R. Eirich** (*Cambridge*) (*communicated*) : I should like to draw your attention to one aspect of the problem and to a corresponding experimental possibility which so far has only been touched upon, namely, the action of drugs on protein-structure.

Some years ago McFarlane finally established the existence of pathological serum proteins and investigated their molecular weights in the ultra-centrifuge. Independently we investigated some of the other physico-chemical changes which become apparent in the human serum under pathological conditions. We found very marked and characteristic differences in the course of salt formation and viscosity when the serum was titrated with acids and alkalis, indicating changes in reactivity and molecular volume similar to those which manifest themselves at an early stage of denaturation.

One explanation for this would be to assume structural changes in the ordinary albumins and globulins due to the presence in the blood-stream of parasites or of the products of an abnormal metabolism in the affected part of the host. An alternative possibility would be to connect the changed behaviour with the appearance of new proteins globulin-like in character (antibodies) under the stimulus of pathological conditions (antigens). According to Pauling both phenomena are correlated.

Interesting information as to the action of drugs in this phase could be obtained by comparing, by the viscosity and conductivity method, the influence of chemotheropeuticals on the serum *in vivo* and *in vitro*. We began such experiments with promising results, but could not continue owing to war-time conditions, and I wonder why this line of approach has not been incorporated into one of the existing research programmes. There are obviously a number of interesting aspects in such an approach :

(1) One ought to be able to differentiate between drug action in the cell and an eventual one during the preceding circulation.

(2) If there is any immediate action on the serum proteins beyond their carrier function this might, in view of the comparatively less complicated nature of the system, provide valuable clues as to the principles of action.

(3) Again, if the serum proteins are affected following a cellular action of the drug, this might throw some light on Bergmann's hypothesis that the intracellular proteinases play a synthetic role *in vivo*. Proteinases apparently lose their specificity in the presence of peptide mixtures, which, it has been suggested, might be the way foreign proteins change the normal course of protein synthesis. If changes in the serum follow only after drug action in the tissue, this action can be positively identified as taking place in the interior of the cell, and becomes also to some extent specified in its character.

(4) Moreover, rectification of a faulty protein-synthesis or structure might have a direct bearing on the enzymatic activity itself, as its protein component might have been affected.

# INTRODUCTORY ADDRESS.

## PART II. PHYSICO-CHEMICAL ASPECTS.

By Professor E. K. Rideal, F.R.S.

*Received 13th September, 1943.*

To commemorate the memory of Sir William Hardy the Faraday Society holds from time to time discussions in which an endeavour is made to bring together people who are interested in some particular sphere of biology and those chemists and physicists who are endeavouring to extend our knowledge of the physico-chemical principles involved in the reactions of organised matter.

A number of attempts have been made to explain the mechanism of drug action on simple principles; one may mention the partition principle of Overton, the capillary view of Traube and the specific group concept of Ehrlich as typical. There are many apparent exceptions to such isolated principles. It may be pertinent to the present enquiry to suggest certain implications in the generally accepted concept that between action and response there is likewise implied a passage, or passages, of material from source to sink. We can depict diagrammatically as follows some simple process which involves such a sequence or chain of events.

$$
\begin{tikzcd}
 & \text{Enzyme A, Co-enzyme A} \arrow[dr] & & & & \\
X \arrow[ur] \arrow[dr] & & Y & \text{Phase II} \arrow[r, "Y"] & \text{Enzyme B} \arrow[r] & Z \\
 & a \arrow[r] & \alpha \arrow[u] & & &
\end{tikzcd}
$$

In the above scheme the reactant X can undergo change to the product Y by alternative paths either through the enzyme system A or the two enzyme systems *a* and α. The product Y has then to pass through a separate phase II which may be complex, *e.g.* a membrane. Y subsequently undergoes change to the product Z by means of the enzyme system B. We may imagine that drug " response " is obtained when the rate of production of product Z is affected. It is clear that this may be brought about in a number of ways. The most direct method is to render the enzyme system B inoperative, by destruction of the protein, by selective adsorption on the prosthetic portion of the enzyme, by reaction with and removal of the co-enzyme or by alteration of the composition, *e.g.* the $p$H of the medium B to stop the enzyme activity. A " delayed " response which eventually will be complete will be obtained if both the enzyme systems A and α or *a* are rendered inoperative by the drug. Clearly if only A or either of the enzymes α or *a* are rendered inactive the drug response will be only partial. Change of the medium of phase I may affect the velocity of reactions of A, α and *a* unequally and the rate of production of Y and thus of Z may show an increase and not a decrease.

Apart from direct action on the enzyme systems which includes possible reaction with the enzyme, co-enzyme or medium, the drug may affect the permeability of the phase II to the product Y or whilst affecting the enzyme system B can only obtain access to it by passage through the phase II. It is consequently important to investigate the separate mechanisms which come into operation under these different conditions.

It is now well established that a number of drugs react directly on enzymes. Here we can distinguish between two distinct types of action; those drugs which react generally with proteins, *e.g.* colloidal silver or mercuric ions and complex mercurials resulting in the coagulation of the

protein and the loss of the necessary spatial configuration of the prosthetic group; and those drugs which are more specific to the enzyme and its carrier. Some quite tentative conclusions can be drawn concerning the specificity of such interactions. In many cases the interaction between drug and enzyme takes place at more than one point. Again, in general, one of these attachments possesses a larger interaction energy than the others. The "strong" attachment is in many cases a "hydrogen" bond. The electron donor group, *i.e.* the group which reacts with the acceptor hydrogen to form the bond may be situated either on the drug or on the substrate. In some cases the hydrogen bonding may be a distinct and separate phenomenon. One of the simplest of these reactions is to be noted in the staining of proteins by dyes. Thus in the case of the coloured wool dyes the hydrogen ion acceptor is the $—COO^-$ group on the side chain of the wool fibre. The peculiar and strong adsorption of acids is due to the fact that in contrast to other ions the hydrogen ion combines with the $—COO^-$ ion to yield a covalent product whilst the other cations remain ionic. The large coloured anion is then adsorbed on another site on the wool macromolecule. We shall return to discuss in more detail the interaction of these large anions but we may note in passing the significant fact that such ions are more strongly adsorbed than inorganic anions, even of greater charge, such as the $SO_4''$. Similar considerations apply to the "dyes" containing large organic cations (*e.g.* the acridine and triphenylmethane series).

More generally the hydrogen bonding involves the whole of the molecule of the drug and not merely the indirect action due to adsorption of an electric charge.

We can identify many electron donating groups both in enzymes and drugs, thus the $>O$ group in ethers, ketones, amides and esters, the $\equiv N$ group in aromatic amines and the $=S$ group in sulphides.

The drug or substrate supplying the hydrogen to form the bond must have a particularly labile hydrogen. We have already noted the strong reactions of the free solvated hydrogen ions, we have also to consider especially the phenolic hydrogen group, and the rather unique —CO—NH— group where mutual reaction is possible since the group is both donor and acceptor in character. Weaker groups are found in the alcoholic hydroxyl and the weakest is the $\equiv CH$ group. The stability of the hydrogen bond can be regarded as determined in part either by the extent of resonance, possibly between the two forms

$$R_2O \quad HC\begin{matrix}X\\Y\\Z\end{matrix} \rightleftharpoons R_2\overset{+}{O} \quad H\overset{-}{C}\begin{matrix}X\\Y\\Z\end{matrix}$$

or by simple considerations of straight dipole interaction

$$\text{e.g.} \qquad >\overset{+}{C}\overset{-}{O} \quad \overset{-}{H}\overset{+}{C}\begin{matrix}X\\Y\\Z\end{matrix}$$

Reactive $\equiv CH$ molecules will be found in those compounds in which suitable activating radicals are introduced into X, Y, and Z. It is interesting to note that the halogens are included in such groups. Chloroform is relatively highly reactive; the bond strength to a ketone being some 5·0 K. cal./gram. mol. Even greater values are found by fluorine substitution as in $HCCl_2F$.

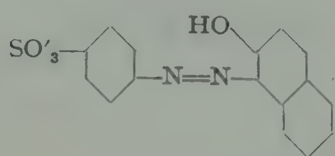
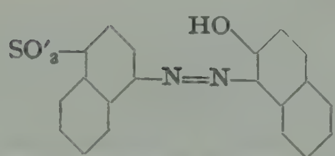
In some cases two or more hydrogen bonds can be formed with the same reacting molecules giving, not only a stronger adhesion, but a great measure of spatial selectivity. I might mention the tanning agents, the 44′dihydroxy stilbene derivatives, the $\alpha\omega$-di-imidines and the *p*-aminobenzoic derivatives, as examples.

The case of chloroform is interesting since this drug does not affect appreciably the surface tension of water, being an exception to the Traube

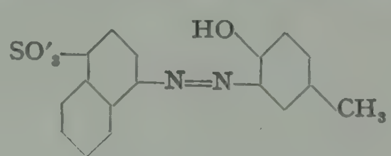
rule. The interaction of chloroform with water and alcohols is small since the mutual interaction between the water or alcohol molecules themselves is so great. We must consequently pay some attention to the competitive action of water in drug action and examine the methods by which this competition is overcome.

The non-polar, hydrocarbon or lipoid portion, both of the drug and of the substrate, provide the important secondary attachments by which this competitive action is reduced and by which also some degree of spatial specificity is introduced. In the adsorption of organic acids we have seen that the unique character of the hydrated proton in forming a covalent linkage with the —COO′ of the protein side chains results, from considerations of charge alone, in adsorption of the anion on a neighbouring site, preferably where the positive charge is situated. The organic anions are more strongly adsorbed than the inorganic ions even of higher valency and it is to the non-polar portions both of the anion and of the substrate, *i.e.* the side chains in a protein and the lipoid constituent in a lipo-protein, that this strong adsorption is due.

Recently Mr. Gilbert has been examining in my laboratory the free energies of adsorption on to proteins of a number of organic compounds of known constitution, and although this field of investigation is as yet in a very embryonic state some of the results he has obtained are very suggestive. Thus he finds :—

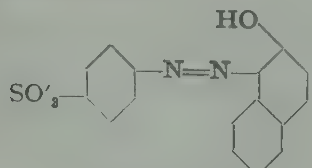
| Anion. | Affinity in K. Cal. at 100° C. |
|---|---|
|  | − 1·52 |
|  | − 0·19 |

From examination of a large number of anions it is found that the contribution to the interaction of a benzene ring can be expressed as effecting an average increment of 1·34 K. cal./ring. If we compare the above with the following value for

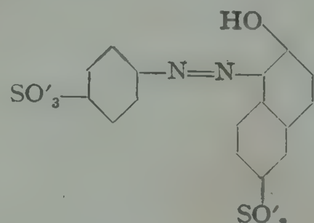


− 2·21 K. cal.

we find an increment of 0·7 K. cal. from which we may infer that adding a methyl group increases the affinity by 1·34 − 0·7 or ca. 0·64 K. cal./mol. $CH_3$. We can in a similar manner examine the decrease in affinity caused by the introduction of a polar group. Thus Mr. Gilbert from examination of systems such as :—



− 1·52 K. cal.



− 2·55 K. cal.

finds that the introduction of further —$SO_3^-$ groups in spite of their negative charges brings about a reduction in intrinsic affinity in this case of ca. $-1{\cdot}03$ K. cal./mol. $SO_3^-$. Multi substitution by —$SO_3^-$ groups always reduces the affinity although the extent of the reduction is dependent on the position of the substitution. Such substitutions may, however, be desirable in order to introduce solubility into the drug.

The spatial orientation of the apolar portions of the adsorbing molecules play an important part in the magnitude of the affinity of adsorption. This has been demonstrated in simple cases, such as cis and trans azobenzene on wool. A quantitative assessment of the " pattern " of apolar portions of a drug would seem to be capable of realisation.

The chromatographic method of purification which has proved so successful in preparative organic chemistry can be readily extended to serve as a most valuable method of examining and evaluating these affinities of adsorption of series of drugs in which progressive variation is made in their constitution.

We have noted in general the factor of drug accessibility has to be considered and this is depicted in the diagram by the introduction of the separate phase II, which may be regarded as a membrane which (in the diagram) must be permeable both to the product Y and any drug affecting the enzyme system B. The membrane is bounded by two interfaces, and the nature of the actions which can occur at interfaces are thus of fundamental importance. Dr. Schulman has dealt with several aspects of these reactions on which we have been engaged for many years. I will in consequence merely make a few comments on this point. The phenomenon of monolayer penetration where on injection of a reactant underneath a monolayer a reaction occurs in the sense that the injected molecules penetrate the monolayer and form a one-one complex with a resulting increase in area or in pressure of the monolayer, is only noted where there is both head group interaction frequently, as Dr. Alexander has shown, of the hydrogen bond type and, in addition, adlineation and interaction between the tails or non-polar portions of the two reactants. This represents a modification of both the Traube and Overton concepts. Furthermore, the mixed monolayer may, in several cases, be displaced, " peptised " or " deterged " from the surface by the added reactant and driven into the substrate as a complex, *e.g.* a lipo-protein. In view of the completeness of this type of reaction it seems improbable that the phase II can ever consist of a single monolayer and in consequence natural membranes must be thicker than a monolayer.

Further evidence is found to support this view, since monolayers and even multilayers of some thickness of proteins do not apparently offer any resistance to the flow of ions through them. These experiments are worth extending to include monolayers and multilayers of the lipoids and lipo-proteins concerning the permeability of which our information is much less complete. Experiments on cholesterol protein monolayers support the view that the cholesterol is anchored to the protein at specific points and that the injection underneath such a lipo-protein monolayer of both protein penetrating and lipoid penetrating reagents results in interaction whereas under pure protein or pure lipoid monolayers the reactions are selective.

These complexes formed by head and tail interaction are clearly important in many biological processes ; they may act as carriers for otherwise non- or difficultly-transportable reactants ; they may remove a reactant from the system or may displace a reactant from a substrate.

The evidence from monolayer properties and behaviour suggests that phase II must in general be considerably thicker than a monolayer, it must consist of a real membrane and contain lipo-protein. The fact that cholesterol has definite points of attachments to a protein monolayer leads one to infer that the lipo-protein membrane possesses some definite structure laminated in character ; that this structure is always as uniform

as the investigations of Schmitt and his co-workers on the nerve myelin sheath suggest is unlikely ; but it is clear that such a composite membrane will be permeable to polar, apolar, and heteropolar molecules, the relative permeability of each type being dependent on the phase volume relation and orientation factor of the two constituents. Such a view suggests that the properties of the membrane as far as permeability are concerned are comparable to those which a mozaic membrane would possess. Evidently, either phase can be extended by suitable solvents resulting in the increased permeability for lipoids or non-lipoids as the case may be and passage which can be termed interfacial in which both the protein and lipoid phases participate can be envisaged. Several aspects of this effect are dealt with by Dr. Hurst who considers, *inter alia*, the mode of action of carriers and adjuvants in insecticides.

# CHEMICAL CONSTITUTION AND PHARMACOLOGICAL ACTION.

By H. R. Ing.

*Received 9th August*, 1943.

The first systematic study of the relations between chemical constitution and pharmacological action was made by Crum Brown and Fraser [1] in their classical work on the metho-salts of alkaloids. They postulated that the physiological action $\Phi$ of a drug was a function of its chemical constitution $C$, in which term they included both the structure and the energy relations of the molecule ; they even put this relation into mathematical form : $\Phi = \mathrm{f}(C)$. This extreme physicochemical view would hardly be accepted to-day since it neglects the highly complicated nature of the physiological situation in drug action. The importance of the physiological situation was stressed by Ehrlich, and although Ehrlich's so-called side-chain theory is now regarded as too simple and too rigid, modern views are more closely akin to his general theory of drug action than to the ideas of Crum Brown and Fraser. Ehrlich would have classified drugs in terms of their capacity to combine with protoplasmic constituents rather than pharmacological actions in terms of chemical structure.

Modern views are both less ambitious and less precise than those of Ehrlich ; they may be summarised in two rather vague principles : (1) Similarity of chemical structure in drugs may be expected to involve similarity of pharmacological action ; this is the working hypothesis of the organic chemist ; and (2) the action of a drug depends upon the way in which it impinges on the normal chemical mechanisms of the tissues. Both principles are to be found in Ehrlich's writings.

A modern version of Ehrlich's general theory of drug action has been put forward and critically examined by A. J. Clark [2] in his "General Pharmacology". Clark assumes that drug molecules combine with specific receptor substances, which in most cases appear to be situated on cell surfaces ; the physiological action of the drug is regarded as occurring subsequently to, but in consequence of, this drug-receptor combination. The nature of the drug-receptor combination is unknown, but as it is often reversible, it is regarded as analogous to the enzyme-substrate combination assumed to precede enzyme activity.

[1] Crum Brown and Fraser, *Trans. Roy. Soc.*, Edinb., 1868-9, **25**, 151.
[2] Clark, "General Pharmacology," in Heffter's *Handbuch der experimentellen Pharmakologie*, 1937.

In his examination of the evidence Clark concluded " that a somewhat surprisingly large proportion of the more accurate quantitative data can be interpreted as the expression of a chemical reaction between the drug and specific receptors " and consequently it may be of value to consider to what extent the theory can be used to interpret the data on the relations between chemical constitution and pharmacological action ; it will be the purpose of this article to make the attempt.

It may be noted first that the receptor theory provides an intellectual link, otherwise missing, between the diverse concepts of structure and action ; ideally the relation to be sought is one between the structures of the drug and receptor molecules. The first general principle mentioned above should be that similarity of chemical structure in drugs may be expected to involve combination with the same receptors. It is our complete ignorance of the chemical nature of the hypothetical receptors which forces us to look for relations between the structure of drug molecules and the physiological effects presumed to flow from the drug-receptor combination. It will also be noticed that the receptor theory only pushes the problem of drug action a stage further back since it provides no explanation of the physiological efficacy of the drug-receptor combination. At the same time the theory has solid advantages ; it offers the possibility of relating the structure of drugs to their action in chemical terms, it brings drug action into line with the theory of chemical transmission and draws an analogy between drug action and enzyme action which may well prove fruitful to the study of both.

**Pharmacodynamic Groups.**—In devising synthetic drugs the organic chemist frequently uses a known drug as a model and, relying on the principle that similarity of structure may involve similarity of action, tries to discover what structural features must be retained in order to preserve the typical activity. The very large body of work on these lines has made it clear that the pharmacological properties of drug molecules can frequently be shown to depend upon particular structural features, which may be regarded as pharmacodynamic structures or groups. The evidence for such groups is of three main types :

(1) Alteration or removal of particular groups in a drug molecule may lead to drastic diminution or even complete disappearance of a particular pharmacological response ; *e.g.* the integrity of the unsaturated lactone ring in the cardiac glycosides is essential for the retention of the typical action on the heart and as Ehrlich discovered the benzoyl group is essential to the local anæsthetic action of cocaine.

(2) Conversely, alterations in structure which leave the typical action of a drug molecule unchanged reveal inessential structural features, *e.g.* the carbomethoxy group in cocaine and the detailed structure of the aliphatic side-chain in vitamin D.

(3) The synthesis of new structures based on evidence of types (1) and (2) has supplied convincing evidence of the existence of pharmacodynamic groups in certain classes of drugs ; *e.g.* it can be predicted with reasonable certainty that molecules embodying the structural unit, $R_1R_2N\,.\,(\dot{\underset{\cdot}{C}})_n\,.\,O\,.\,CO\,.\,Ph$ where $n = 2, 3, \ldots$, will possess local anæsthetic properties. A striking illustration is provided by the observation that the attachment of a $\beta$-benzoylethyl or a $\gamma$-benzoylpropyl group to the secondary nitrogen atom of the alkaloid cytisine confers considerable local anæsthetic properties on the new molecule, while the powerful nicotine-like actions of cytisine itself completely disappear.[3]

Pharmacodynamic groups have also been identified in atropine and physostigmine and synthetic substitutes for these drugs synthesised. With a few exceptions, Crum Brown and Fraser's original generalisation that quaternary ammonium salts, and onium salts in general, exert a

[3] Ing and Patel, *J. Pharmacol.*, 1937, **59**, 401.

curare-like action remains true; the onium cation must be regarded as the first pharmacodynamic group to be discovered.

Pharmacodynamic groups are the lineal descendants of Ehrlich's anæsthophore groups, toxophore groups, etc., and their occurrence finds a simple interpretation in terms of the receptor theory; the receptors, which must be regarded as adapted to some normal physiological process, will combine with and respond to strictly limited types of molecules. The more highly selective the action of the drug the more probable becomes the hypothesis of specialised receptors which respond only to molecules with precisely defined structural features. Certain classes of drugs, such as autonomic drugs and cardiac glycosides, exert a selective action comparable only with that of hormones and chemical transmitters and to account for the action of the latter some form of receptor theory appears to be necessary.

## Drugs of Diverse Structure and Similar Pharmacological Action.

Although certain pharmacodynamic groups may be established, similar pharmacological properties may be shown by drugs of quite different structure; thus local anæsthetic properties are displayed by drugs which do not conform to the dialkylaminoalkyl benzoate type, *e.g.* benzyl alcohol, quinine and certain anti-malarials of the plasmoquin type.[4] Such examples do not invalidate the relations established between structure and action among local anæsthetics of the dialkylaminoalkyl benzoate type so long as it is admitted that local anæsthesia may be achieved by a variety of mechanisms. That the same physiological result can be achieved by different mechanisms is well established, *e.g.* the dilatation of the pupil by atropine and by ephedrine and the parasympathetic actions of acetylcholine and eserine, and consequently the assumption that the same physiological result may be produced by different mechanisms can often be made without impropriety. The diversity of chemical antiseptics is not surprising; the living cell is an extremely complex system and a drug which inhibits any one of a (probably large) number of vital chemical processes may be expected to lead to the death of the cell. Moreover, the cell has only a limited number of ways of responding to stimuli; it can only display its normal types of activity in increased or diminished intensity. Since the normal activities of a cell certainly involve a large number of consecutive chemical reactions, drugs may attack different points in this chain of chemical events and yet produce the same end result.

The difficulty only becomes acute when we consider groups of drugs of diverse structure which possess similar and highly selective actions occurring at well-defined sites in the body. The parasympathomimetic drugs, acetylcholine, muscarine, physostigmine and pilocarpine constitute such a group. Here again, more detailed pharmacological analysis may demonstrate, as has been done for physostigmine, that different mechanisms are involved.

Closer definitions of similarity and diversity of structure are made necessary by the receptor theory, and in this connection more consideration should be given to changes in physical properties accompanying structural changes. In their classical work on sympathomimetic amines, Barger and Dale[5] observed the qualitative similarity in action of isoamylamine and adrenaline; on chemical grounds it is difficult to imagine that a simple aliphatic amine can form an effective combination with the same receptors as the more complex amphoterric substance adrenaline;

[4] *E.g.*, 8(γ-diethylamino-ββ-dimethylpropylamino)-6-ethoxyquinoline; Bovet, *Arch. internat. Pharm. Therap.*, 1931, **41**, 103.

[5] Barger and Dale, *J. Physiol.*, 1930, **41**, 19.

and when the great difference in the intensity of the actions of these two substances is borne in mind, the view that they combine effectively with the same receptors involves a surprising discrepancy between the qualitative and the quantitative discrimination of the receptors. Attempts to relate structure and action among such physicochemically dissimilar drugs is bound to be fruitless; even ephedrine, which has a greater structural resemblance to adrenaline than isoamylamine, must be regarded as physicochemically dissimilar. These "sympathetic" amines are now classified by some workers as sympathicotropic drugs, which are thought to form effective combinations with the adrenaline receptors and which have amphoterric structures closely related to adrenaline, and sympathomimetic drugs which are less closely related to adrenaline in structure and physical properties and are thought to achieve "sympathetic" action by other mechanisms.[6] The view that such amines as isoamylamine and ephedrine are on physicochemical grounds unlikely to form effective combinations with the adrenaline receptors does not rule out the possibility of their forming ineffective combinations; ephedrine is known to inhibit amine oxidase, which can catalyse the oxidation of adrenaline, and also to antagonise adrenaline (see below).

Another example of drugs of diverse structure and similar pharmacological action is provided by the aliphatic narcotics, which include a large variety of structural types, *e.g.* hydrocarbons, alcohols, ethers, amides, urethanes, sulphones, etc. These drugs appear to achieve their effect by modifying the physicochemical conditions of cells, and their action is regarded as depending not on pharmacodynamic groups but on certain physical properties shared by all classes of these compounds.

## Drugs of Similar Structure and Diverse Pharmacological Action.

The observation that the action of a particular drug may be antagonised by another drug of similar structure occurs fairly frequently and finds a simple interpretation in terms of the receptor theory. If it be assumed that both drugs combine with the same receptors, but that only one of the drug-receptor combinations is physiologically effective, then the ineffective drug-receptor combination will reduce the activity of the effective drug by limiting the number of free receptors available to it. This theory involves the assumption that the structural requirements of the receptor for combination with a drug are less exacting than those for the production of the characteristic physiological response. There is considerable justification for this assumption because the inhibition of enzymes by compounds structurally related to their normal substrates is well established. Thus, ephedrine inhibits the oxidation of adrenaline by amine oxidase *in vitro*;[7] presumably it can combine with the enzyme, but unlike adrenaline it is not oxidised. Similarly, ephedrine has been found to antagonise a variety of the actions of adrenaline, *e.g.* on the dog's blood pressure, rabbit's intestine, etc.,[8] and these results can be accounted for by the assumption that ephedrine combines with the adrenaline receptors but that the combination is physiologically ineffective.

An even more striking example was studied by Raventos,[9] who found that the higher members of the homologous series of cations $R\,.\,\overset{+}{N}Me_3$ ($R = C_7H_{15}$, $C_8H_{17}$, $C_{16}H_{33}$) antagonised the muscarine-like action of the lower members ($R = CH_3$, $C_2H_5$, $C_3H_7$, $C_4H_9$, $C_5H_{11}$) on the frog's auricle.

[6] *Cf.* Gaddum and Kwiatkowski, *J. Physiol.*, 1938, **94**, 87.
[7] Blashko, Richter and Schlossmann, *Biochem. J.*, 1937, **31**, 2187.
[8] For references, see Gaddum and Kwiatkowski, *loc. cit.*[6].
[9] Clark and Raventos, *Quart. J. exp. Physiol.*, 1937, **26**, 375; Raventos, *ibid.*, 1938, **27**, 99.

The lower members acted additively with acetylcholine and were antagonised by atropine; the higher members antagonised both acetylcholine and the lower members. Moreover, the doses of a higher member (*e.g.* $C_8H_{17}\overset{+}{N}Me_3$) required to reduce the effect of equiactive doses of $Me_4\overset{+}{N}$ and acetylcholine by a given amount were similar, although equiactive doses of $Me_4\overset{+}{N}$ and acetylcholine were in the ratio 1000 : 1. These facts can be explained by the assumption that all the cations $R\overset{+}{N}Me_3$ combine with the acetylcholine receptors but that the combination is physiologically ineffective for the higher members.

It is interesting to note that the first recorded example of onium salts antagonising acetylcholine was only recognised as such in recent years; it is the curariform action of onium salts [10] discovered by Crum Brown and Fraser.

The occurrence of enzyme inhibition by a competitor of structure related to the normal substrate has been recognised recently as of great importance in bacterial chemotherapy. Since the original observation of the mutual antagonism of *p*-aminobenzoic acid and sulphanilamide *in vitro* [11] and *in vivo*,[12] several other pairs of mutually antagonistic substances in bacterial metabolism have been recorded, *e.g.* nicotinic acid and pyridine-3-sulphonic acid,[13] pantothenic acid and pantoyltaurine,[14] etc., and there is little doubt that other examples will be discovered.

## Quantitative Considerations.

The influence of structural changes on the intensity of drug action provides the most difficult problems in any attempt to relate structure and action. Knowledge in this field is handicapped by the difficulty of measuring most pharmacological actions accurately and is complicated by the use of different methods by different authors.

It may be of value at the outset to consider how differences in intensity of action are to be interpreted in terms of the receptor theory. The intensity of a drug action will on this theory depend at any moment on the number of drug-receptor combinations, and for any given concentration of drug, this number will be determined by the ease with which the drug-receptor combination is formed. For a reversible drug reaction the relation between concentration $c$ and action $y$ should follow a rectangular hyperbola, $Kc = y/100 - y$, where $y =$ percentage of the maximum possible action. Clark [2] has discussed the applicability of this equation in detail; a similar equation holds for the reversible inhibition of enzymes by metals [15] and dyestuffs.[16]

The drug-receptor combination must be regarded as leading to an all-or-none type of response; if this were not true, a weaker drug would partially antagonise a stronger when both were used together. Raventos [9] found that this did not occur with combinations of acetylcholine and onium cations such as $Bu\overset{+}{N}Me_3$ on the frog's heart; although $Bu\overset{+}{N}Me_3$ had only about a hundredth of the activity of acetylcholine, it acted additively with acetylcholine when combinations of the two drugs were used. This result implies that equal intensities of action involve equal

[10] Ing, *Physiol. Rev.*, 1936, **16**, 527.

[11] Woods, *Brit. J. exp. Path.*, 1940, **21**, 74.

[12] Selbie, *ibid.*, p. 90.

[13] McIlwain, *ibid.*, p. 136.

[14] Snell, *J. Biol. Chem.*, 1941, **139**, 975; **141**, 121; Kuhn, Wieland and Müller, *Ber. dtsch. chem. Ges.*, 1941, **74**, 1605; McIlwain, *Biochem. J.*, 1942, **36**, 417.

[15] Myrbäck, *Hoppe-Seyler's Z.*, 1926, **158**, 160.

[16] Quastel and Yates, *Enzymologia*, 1936, **1**, 60.

numbers of combinations of the different drug molecules with the receptors, but the concentrations needed to produce equal numbers of combinations may vary over a very wide range.

Although the individual reaction between drug molecule and receptor must be regarded as producing an all-or-none response, the total physiological effect observed will usually appear as a graded response because of the large number of receptors involved.

The ease of combination of a drug with specific receptors may be expected to depend upon a large number of factors, *e.g.* stereochemical configuration, size and shape of the molecule and the disposition of the pharmacodynamic groups, the nature of acidic and basic groups, etc.

## Stereochemical Specificity.

Stereoisomeric drugs often differ in the intensity of their action. The most striking examples are those optical enantiomorphs, *e.g.* *l*-adrenaline and *l*-hyoscyamine, which are twice as active as the corresponding racemic compounds. The subject was thoroughly investigated by Cushny,[17] who argued that a clear differentiation in the activities of optical enantiomorphs indicated that the drug combined chemically with an optically active tissue constituent and that the different activities were to be ascribed to different physicochemical properties in the compounds so formed. Other factors might also be involved, *e.g.* more rapid destruction of one enantiomorph by enzymic action in the body.

Cushny's views are difficult to reconcile with the receptor theory because he assumed that the intensity of the physiological action was determined by the nature of the combinations which each enantiomorph formed with the same tissue constituent; but on the receptor theory both enantiomorphs must form equally effective combinations with the receptors. If this were not so, the less active enantiomorph would partially antagonise the more active when both were present. Experiment shows, however, that optical enantiomorphs act additively when used in combination; thus Cushny [17] found that *l*-hyoscyamine was 15-20 times as active as *d*-hyoscyamine on the dog's salivary gland and twice as active as atropine; *l*-hyoscine was 16-18 times as active as *d*-hyoscine and twice as active as *dl*-hyoscine; *l*-adrenaline was 12-15 times as active as *d*-adrenaline in its vasoconstrictor action and twice as active as *dl*-adrenaline. These results agree with the requirements of the receptor theory, but it must be admitted that the quantitative data are scanty and a closer examination of the additive properties of optical enantiomorphs is desirable.

On the receptor theory the difference in activity of optical enantiomorphs will depend upon the ease with which each isomer combines with the receptors. This conclusion is remarkable but appears to be inescapable if they act additively. On the other hand, the view that the ease of combination with receptors is determined by stereochemical configuration appears to be reasonable in the case of stereoisomers which are not optical antipodes.

## Homologous Series.

A study of the activities of homologous drugs is of interest because the structural changes involved are of the simplest character. Two main types may be distinguished: (1) Homologous series in which the activity increases regularly as the series is ascended, *e.g.* the aliphatic narcotics, and (2) series in which the activity increases to a maximum and then declines.

[17] Cushny, *Biological Relations of Optically Isomeric Substances*, Baltimore, 1926.

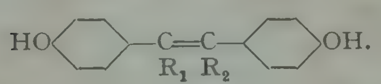
(1) When the logarithm of the narcotic concentration of homologous compounds is plotted against the number of carbon atoms a linear relation is obtained. Ferguson [18] has pointed out that with such a series all the physical properties like solubility, vapour pressure, surface tension, etc., which depend upon a distribution between two phases, also change logarithmically with the number of carbon atoms. It is generally agreed that narcotic activity depends upon a distribution equilibrium between the medium bathing the cells and the biophase (whatever it may be) in which the drug has its effect, but the prolonged controversy between the differential solubility and the adsorption theories of narcosis reflects the difficulty of deciding between properties which vary from one member to the next in exactly the same way. Which physical property determines narcotic activity cannot be decided by consideration of the relations between activity and physical properties in one series; the effects produced by members of different homologous series must be correlated.[19] This problem still awaits solution.

The logarithmic decrease in narcotic concentration in a homologous series proceeds until a member is reached which exerts the same narcotic effect as the lower members only when present as a saturated vapour or solution; beyond this member, further members will be inactive or only feebly active.

(2) The commonest type of variation within a homologous series is that the activity rises to a maximum as the series is ascended and then declines. A striking example is provided by the oestrogenic activity of the 4:4′-dihydroxy-dialkyl stilbenes,[20] which show maximum activity for the diethyl member (Table I).

TABLE I

OESTROGENIC ACTIVITY OF DIHYDROXY-DIALKYL STILBENES.



| $R_1$. | $R_2$. | Rat Units Per Gram. |
|---|---|---|
| H | H | 140 |
| H | Et | 5,000 |
| Me | Me | 40,000 |
| Me | Et | 1,000,000 |
| Et | Et | 3,000,000 |
| Et | *n*Pr | 300,000 |
| *n*Pr | *n*Pr | 50,000 |
| *iso*Pr | *iso*Pr | 20,000 |
| *n*Bu | *n*Bu | 5,000 |

Similar results have been observed with alkyl phenols and cresols,[21] alkyl resorcinols,[22] alkyl hydrocupreines,[23] etc.; among antiseptics of this type the position of the maximum in the series may vary with the type of organism used.

Barger [24] argued that the activity in such series must represent the summation of two opposing effects, which must be physical in character; thus, in the aliphatic primary amines, which show maximum pressor action at the *n*-hexyl member,[5] he suggested that the two opposing effects might be the decline in basicity and the increase in surface tension as the series is ascended. The view that the ease of formation of the drug-receptor combination depends upon an optimal balance of two opposing effects is attractive, but it is difficult to see how it could be tested.

Barger also suggested that when two pharmacodynamic groups in a drug molecule are separated by an alkyl chain, the length of the chain may determine the ease of combination with receptors, *e.g.* in local

[18] Ferguson, *Proc. Roy. Soc., B*, 1939, **127**, 387.
[19] Meyer, *Trans. Faraday Soc.*, 1937, **33**, 1067.
[20] Dodds, Goldberg, Lawson and Robinson, *Proc. Roy. Soc. B*, 1939, **127**, 142.
[21] Coulthard, Marshall and Pyman, *J. Chem. Soc.*, 1930, 280.
[22] Leonard, *J. Amer. Med. Ass.*, 1924, **83**, 2005.
[23] Morgenroth, *Biochem. Z.*, 1914, 11.
[24] Barger, *Some Applications of Organic Chemistry to Biology and Medicine*, New York, 1930.

anæsthetics, maximum activity is usually shown by members in which the dialkylamino and benzoyl groups are separated by a three-carbon chain.

In a recent study of the bacteriostatic properties of polymethylene diamines, diamidines, diguanidines and di-isothioureas Fuller [25] found that maximal activity was usually reached when the polymethylene chain had 14 to 18 carbon atoms. Interpretation of the results is, however, made difficult by his observation that the position of the maximum in all series except the di-isothioureas varied with the medium; the maximum occurred at a shorter chain length for organisms in serum than for the same organisms in broth.

The assumption that any homologous series of drugs will show maximum activity for one member is a useful working hypothesis for the organic chemist but not all homologous series display the satisfactory regularity of the examples already quoted; thus the relative activities of homologous choline esters exhibit remarkable relations (Table II).

TABLE II

RELATIVE POTENCIES PER MOLECULE OF CHOLINE ESTERS.[26]

(Acetylcholine = 100 in each case.)

| Ester. | Rabbit Intestine. | Frog's Rectus Abdominis. | | Leech. | Rabbits Blood Pressure. |
|---|---|---|---|---|---|
| | | Without Eserine. | After Eserine. | | |
| Acetyl . . . | 100 | 100 | 100 | 100 | 100 |
| Propionyl . . | 3 | 550 | 450 | 45 | 4 |
| Butyryl . . | 0·24 | 90 | 115 | 90 | 0 |
| Valeryl . . . | 0·20 | 25 | 30 | 0·9 | 0 |

Homologous series are also known in which one member (other than the first) has minimum activity; *e.g.* minimum curariform activity occurs for R = Et in the series $R_4\overset{+}{N}$, $R_4\overset{+}{P}$ and $R\overset{+}{N}Me_3$, where R = Me, Et, Pr and Bu. This effect is not caused by any intrinsic properties of the ethyl group because in the arsonium series $R_4\overset{+}{As}$ the least active member is $Me_4\overset{+}{As}$, and $Et_4\overset{+}{As}$ is equal in activity to $Me_4\overset{+}{N}$; also in the alkylquinolinium series the metho-salts are less active than the etho-salts.[27] These examples are peculiarly puzzling because among simple onium salts the curariform activity is remarkably independent of detailed chemical structure and appears to depend primarily on the ionic character of onium cations.[10]

## Conclusion.

The receptor theory provides a useful intellectual framework for the consideration of the qualitative aspects of the problems presented by the structure and action of drugs; in particular it is the only satisfactory means of accounting for the antagonism of structurally similar drugs. It is less useful for the consideration of the quantitative aspects of the subject because "ease of combination with the receptors" is too vague a concept when the structural requirements of the receptors are unknown.

[25] Fuller, *Biochem. J.*, 1942, **36**, 548.
[26] Chang and Gaddum, *J. Physiol.*, 1933, **79**, 255.
[27] Ing and Wright, *Proc. Roy. Soc. B*, 1933, **114**, 50.

It is possible in certain cases to predict the kind of drug action which will result from structural changes in a drug molecule, but the changes in intensity of action resulting from even the most trivial alterations in structure cannot be predicted.

The receptors must be regarded as highly complex, and specialised receptors may differ in different tissues. Some such assumption appears to be necessary to account for the fact that the qualitative and quantitative actions of drugs differ in different tissues; *e.g.* atropine is the best known antagonist of acetylcholine but it does not antagonise acetylcholine in mammalian voluntary muscle; $Me_4\overset{+}{N}$ acts additively with acetylcholine on the frog's heart but antagonises acetylcholine in voluntary muscle, while Table II illustrates the quantitative irregularity of choline esters in different tissues. Facts of this kind led Clark [28] to conclude that every cell-drug system was a law unto itself. It is, however, a sound rule in studies of the structure and action of drugs to confine attention to one pharmacological action in one tissue and not to expect relations so observed to be necessarily applicable to other actions in other tissues.

It has been tacitly assumed throughout this article that all the receptors in a given tissue are equally accessible to drugs and equally effective in the physiological sense. Both assumptions are rather improbable; it is more likely that the receptors are subject to individual variation. If this be assumed, a reconciliation might be effected between Clark's view that concentration-action curves express the relation between the concentration of the drug and the uptake by the receptors and Shackell's [29] view that they express the relation between the uptake of the drug and its effect on a mixed population of cells.[30]

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## *GENERAL DISCUSSION*

**Dr. Ing** (*Oxford*), in introducing his paper, said: In his Introductory Address Sir Henry Dale criticised the receptor theory on the ground that it added nothing to our knowledge, but was only an alternative way of describing the well-established pharmacological facts. The theory is not meant to be other than an alternative method of describing the known facts, which, however, is thought to have the additional advantages of suggesting new approaches to the problems and of providing a much-needed intellectual link between the diverse concepts of chemical structure and pharmacological action.

In quoting the selective and similar stimulant properties of tetramethyl-ammonium salts and the natural alkaloids, nicotine, cytisine and lobeline, Sir Henry Dale has mentioned one of the most puzzling examples of diverse structure and similar physiological action, but his assumption that on the receptor theory all these drugs must be thought to have affinity for the same chemoreceptors is not necessarily well founded. The more selective a drug action is, the more probable is the assumption that the drug acts in virtue of an affinity for specialised receptors and the less likely that such receptors will combine with drugs of diverse structure. It is not necessary to assume that a highly selective action elicited by a variety of drugs always implies combination with the same receptors, as the well-known example of acetylcholine and eserine illustrates. It may prove to be a merit of the receptor theory that it draws attention to problems, like that quoted by Sir Henry Dale, where closer pharmacological analysis is needed.

[28] *Loc. cit.*,[2] pp. 190-199.
[29] Shackell, *J. Gen. Physiol.*, 1923, **5**, 783.
[30] *Cf.* Gaddum, *Proc. Roy. Soc. B*, 1937, **121**, 598.

**Mrs. Catherine Le Fèvre** (*London*) (*communicated*) : For a few years prior to the outbreak of war, Dr. R. J. W. Le Fèvre and I were investigating at University College, London, the Kerr Electro-Optical properties of organic substances in vapour and solution form. From the results obtained from our experiments, in conjunction with results we also obtained from the measurements of dielectric constants which yielded dipole moment data, we have been able to calculate the optical polarisibility tensor ellipsoids of these substances. For the solvent state, only an approximate of the true polarisibility of a molecule along the three directions—maximum, minimum, and the direction perpendicular to these two—of polarisibility which define the tensor ellipsoid, can, of course, be ascertained. But in the investigation of substances in an homologous series dissolved in a solvent of low Kerr constant, it should be possible to obtain a good qualitative picture, which might throw considerable light on why there appear to be the two main types observed in the activities of homologous drugs. A series, *e.g.* the dihydroxy dialkyl stilbenes in which the activity increases to a maximum and then declines, might possibly be explained by a change in geometric structure due to buckling of the molecule, this would easily be detected by experiments on the Kerr effect.

Further, divergences in the optical polarisibility tensor ellipsoids, which conforms to the geometric shapes of the molecules, in the choline ester series might again throw light on the anomalous relative activities observed, yielding ultimately to information concerning the structural requirements of the receptors.

W./Cdr. Le Fèvre is on active service in the Far East, but he would wish to join me in offering Dr. Ing, through our own investigations post war, any help that we might be able to give in relating polarisibility and pharmacological activity.

**Dr. H. R. Ing**, in reply, said : The measurement of many physical properties involves techniques unfamiliar to the organic chemist and the kind of co-operation between organic and physical chemists which Mrs. Le Fèvre has suggested seems to me to be the best way to advance the subject.

**Dr. D. B. Taylor** (*London*) said : The consideration of the active group of an enzyme as a "receptor" sheds considerable light on the relationship between drug structure and action. For example, the very large effects produced by polar substitution in certain organic molecules on the velocity of their hydrolysis by specific enzymes suggests that even the polar contribution of hydrocarbon side chains may be of importance.

**Dr. D. D. Eley** (*Cambridge*) (*communicated*) : Throughout the Discussion attention has been focussed upon the importance of Van der Waal's forces and related bonds, as distinct from the covalent bond. In support of this view, one contributor in the Discussion has stressed the reversible nature of the drug-receptor combination. While not wishing to contradict this view-point, I would like to point out that the possibilities of covalent bond formation should not be neglected. The formation of covalent bonds between drug and receptor may in principle be expected to introduce more profound changes in the receptor (*e.g.* enzyme molecule) than the mere formation of a Van der Waal's bond, since in the former case the whole pattern of the molecule may be expected to be altered. Of course, the formation of the Van der Waal's complex may be a necessary first step to covalent bond formation, but by itself less likely to introduce profound chemical changes in the enzyme molecule, or other receptor. Secondly, while it is true that many of the well-known reactions of chemistry (such as the $H_2 + I_2$ reaction), in which a rearrangement of covalent bonds occurs, only takes place at high temperatures because of the high activation energy involved, this is by no means universally the case. I would quote the well-known reaction between oxygen and hæmoglobin, thoroughly studied by Hartridge and Roughton. This occurs rapidly and reversibly at room temperature, and one of the early theories assumed it to be a

physical adsorption of the oxygen upon the hæmoglobin colloid. However, during the last twenty years much evidence has accumulated to show that this reaction involves the formation of chemical bonds between the oxygen and the Fe in the hæmoglobin. Recently, L. Pauling and collaborators have shown that the paramagnetic moment of both the hæmoglobin and the oxygen disappear in the reaction to give a diamagnetic oxyhæmoglobin, a fact which indicates an extensive rearrangement of electron levels in the reaction. The reasons for the rapidity of this reaction are still by no means clear, but a few years ago Polanyi and Evans showed in principle how quantum mechanical resonance, that may occur in molecules containing conjugated double bonds, can work so as to lower the activation energy of association reactions. There are also other theoretical mechanisms which may be visualised to explain the rapidity of reactions between proteins, etc., but the need at the present moment is more for an extensive study of a suitably chosen, clearly defined case.

**Dr. H. Hurst** (*Cambridge*) said : The concepts of Ehrlich and Clark that quantitative pharmacological data may be expressed in terms of chemical interaction between drugs and specific receptors, and that drug fixation at these receptors is measured by concentration-action curves are open to the following criticisms :

(1) Owing to the disturbing influence of selective adsorption at functional interfaces in the system, the active concentration of a drug measured in a bulk external drug phase may be much smaller than the actual concentration at the *primary* site of action, *i.e.* the common drug phase/biological phase interface.

(2) The receptor theory is only valid when it is known that drug access is not influenced by a selective diffusion process. The antagonism of drugs which are structurally similar may be partially an expression of molecular interaction or competition for the lipo-protein receptors in a bounding biophase where drug mobility is influenced by " pharmacodynamic " groups and also by less specific hydrocarbon or fat-soluble portions of the drug molecules.

(3) Selective drug access may also account for the irregularity shown by the positions of maximum activity in different homologous series. With increase in the van der Waals' interaction between the hydrocarbon portions of the drug molecules and the lipoid components of the lipoprotein mosaic substrate, two factors influence two-dimensional drug mobility along the functional interfaces of the bounding substrate : (i) an increase in the rate of drug access owing to " carrier action " of the drug molecules which is associated with a decrease in " functional viscosity " of the lipoid loci ; and (ii) a decrease in the rate of drug access owing to the establishment of a *balance* between the polar and non-polar interactions of the drug molecules with the protein and lipoid components of the biological substrate. The establishment of this balance would tend to favour adsorption at the outer surface or layer of the diffusion barrier. The position in a homologous series of drugs at which the competing factors (i) and (ii) become respectively dominant will depend, not only on the stereochemical configurations of the drug molecules, but also on the physico-chemical properties of the acceptor groups in the particular biological tissue or organism. The magnitude and irregularity of the variation in relative activity of the choline esters with increase in chain length can hardly be attributed to corresponding differences in specific drug-receptor combinations based on the somewhat static conceptions of chemical affinity, and may be more readily interpreted in terms of selective diffusion, which varies enormously according to the particular biological component in the system. Further evidence in support of this theory is provided by the fact that the position of maximum activity in a series varies with the carrier medium, suggesting that carrier activity plays an important part in modifying the functional susceptibility of the biological system.

**Dr. H. R. Ing,** in reply, said : The type of drug-receptor combination envisaged in my paper need not be restricted to reactions involving principal valencies, such as salt formation and condensation reactions ; such reactions probably do occur, *e.g.* the reversible formation of thioarsinites by arsenoxides and thiol compounds, but partial valencies may frequently be involved as well as, or even without, principal valencies. Most reversible drug reactions probably occur at cell surfaces, and for such reactions the drug-receptor combination seems to be most usefully regarded as a two-dimensional combination at an interface ; some types of drug specificity seem inexplicable except in terms of surface reactions such as Prof. Rideal has enumerated in his paper. It may be noted in this connection that the nearest analogy to the differential pharmacological activity of some optical enantiomorphs is the differential adsorption of some dia-stereoisomerides.

The importance of the physical properties of drugs has been stressed in several papers submitted to this Discussion, but it appears that some clarification of the significance of such properties to theories of drug action is needed. I am in general agreement with Sir Henry Dale's remarks on this subject in his Introductory Address. What appears to be needed is the same sort of correlation of physical properties with pharmacological activity as the organic chemist has achieved for the structural features of some classes of drugs. It is usually found that several functional groups or structional units must be combined in a drug molecule before a particular pharmacological response can be elicited. In the same way it is probable that drug actions depend upon a combination of physical properties ; it is unlikely that such complex phenomena as drug actions will be found to depend upon one physical property only, however apparently apt to biological situations the property may appear.

# CHEMICAL STRUCTURE OF ARSENICALS AND DRUG RESISTANCE OF TRYPANOSOMES.[1]

BY HAROLD KING.

*Received* 31*st August*, 1943.

The science of chemotherapy was founded by Ehrlich, and in this science one of Ehrlich's most important discoveries was that of drug-fastness or -resistance. This latter subject he developed experimentally and theoretically, and in an inspired manner he adorned the subject by coining technical terms as the vehicle for his thoughts.

Browning, Franke and Roehl,[2] working in Ehrlich's laboratory, were the first to produce drug-resistant strains of trypanosomes, the phenomenon being observed initially with parafuchsin. It was soon shown, however, that drug-resistance could be produced with an arsenical, atoxyl. Such an atoxyl-resistant strain was also resistant to a great number of substituted derivatives of phenylarsonic acid,[3] but infections in mice with this strain could be cured by arsenophenylglycine. Other arsenicals containing the acetic acid radicle contained in arsenophenylglycine, *e.g.* arsenophenylthioglycollic and arsenophenoxyacetic acid, had also the power of curing infections of atoxyl-resistant trypanosomes.[4, 5] Ehrlich and his collaborators made the further important observation that atoxyl-resistant

[1] King and Strangeways, *Ann. Trop. Med. Parasit.*, 1942, **36**, 47.
[2] Ehrlich, *Berl. Klin. Woch.*, 1907, **44**, 233, 341.
[3] Ehrlich, *Ber. deutsch. Chem. Ges.*, 1909, **42**, 36.
[4] Ehrlich, *Arch. Schiffs. Trop.-Hyg.*, 1909, **13** ; Beiheft, **6**, 91.
[5] Ehrlich, *Zeit. Angew. Chem.*, 1910, **23**, 2.

strains were also resistant to a group of dyestuffs, all built on a certain pattern, and represented by the acridine, oxazine, thiazine, selenazine and pyronine dyes, although no resistance was shown to dyestuffs of the trypan-blue type or of the triphenylmethane type.[6] The atoxyl-resistant strains were almost unstained by oxazine dyes whereas normal strains were rapidly stained vitally and then died shortly afterwards. Another visible manifestation of the continuous effect of these dyes on trypanosomes was the production of forms devoid of the kinetoplast. The kinetoplast is not, however, lost during the process of production of atoxyl-resistant forms, and such resistant strains do not, on further treatment with acriflavine, lose the kinetoplast.[7]

A step forward in the controlled investigation of drug-resistance was the introduction by Yorke and Murgatroyd [8] of a method for keeping trypanosomes alive at 37° for 24 hours or more *in vitro*. Using this technique, they found that an atoxyl-resistant strain was resistant *in vitro* to many substituted arsenicals, but not to sodium arsenite, and the natural conclusion was drawn that the resistance was not an arsenic-resistance, but rather a resistance to the substituted phenyl group.[9] These *in vitro* results were confirmed by Yorke, Murgatroyd and Hawking [10] by experiments in the living animal. A curious exception was found in phenylglycinearsonic acid (the pentavalent arsenical corresponding to the exceptional arsenophenylglycine of Ehrlich) in that atoxyl-resistant strains in mice showed no greater resistance to this compound than did normal strains, whereas with other arsonic acids, such as atoxyl, tryparsamide or arsacetin, there was a great difference. Yorke, Murgatroyd and Hawking [11] also demonstrated that strains made fast to atoxyl, arsacetin, tryparsamide, neoarsphenamine or acriflavine were identical.

The nature of the precise constitutional or physiological change which takes place in a drug-fast trypanosome is still unknown. Ehrlich, in explanation of drug-fastness, postulated a diminished affinity of certain chemoreceptors in the parasite for the drug, the non-staining of atoxyl-resistant forms by oxazine dyes being a visible demonstration of this reduced affinity. Yorke, Murgatroyd and Hawking [10] were the first to show in the case of arsenicals that normal trypanosomes removed the reduced tryparsamide from the medium, as shown by a diminished direct trypanocidal action of the circumambient fluid on fresh trypanosomes, whereas drug-fast trypanosomes failed to do so. This was corroborated by Hawking [12] by chemical estimation of the arsenic content of the circumambient fluid and of the trypanosomes. It is again significant that arsenophenylglycine was absorbed by resistant and by normal strains; in addition Hawking made the interesting observation that phenylarsenoxide was also absorbed equally well by normal and resistant trypanosomes, and thus was exceptional among arsenicals of this type.

In 1930 Gough and King [13] showed that a series of aromatic arsonic acids containing acidic groups such as carboxyl or sulpho

$$(AsO_3H_2 \,.\, Ph \,.\, CO_2H \text{ and } AsO_3H_2 \,.\, Ph \,.\, SO_3H)$$

had no curative action on experimental trypanosomiasis in mice, but that when they were converted into amides ($AsO_3H_2$ . Ph . $CONH_2$ and $AsO_3H_2$ . Ph . $SO_2NH_2$) trypanocidal activity appeared in all cases. These

[6] Morgenroth, *Ehrlich's Festschrift*, 1914, 572.
[7] Leupold, *Z. Hyg. Infektskr.*, 1925, **104**, 641.
[8] Yorke and Murgatroyd, *Ann. Trop. Med. Parasit.*, 1930, **24**, 449.
[9] Yorke, *Brit. Med. J.*, 1932, **2**, 668.
[10] Yorke, Murgatroyd and Hawking, *Ann. Trop. Med. Parasit.*, 1931, **25**, 313.
[11] Yorke, Murgatroyd and Hawking, *ibid.*, 1932, **26**, 577.
[12] Hawking, *J. Pharm. Exp. Ther.*, 1937, **59**, 123.
[13] Gough and King, *J. Chem. Soc.*, 1930, 669.

observations were extended by Cohen, King and Strangeways,[14] who found that when the arsenical portion of the molecule was present as the arseno-grouping (—As : As—) then trypanocidal activity was found in five out of seven compounds, although they contained carboxyl groups. Among the active substances was arsenophenylglycine $(CO_2H . CH_2 . NH . Ph . As :)_2$, which played a salient part in Ehrlich's work on types of drug-resistance.

Having available a number of arsenoxides containing carboxyl groups, Miss Strangeways and I thought that it would be of interest to find out whether such arsenoxides act *in vitro* on atoxyl-resistant trypanosomes, and thus belong to the class represented by arsenophenylglycine, or whether they are without action and simulate the atoxyl class.

By employing the method developed by Yorke and Murgatroyd for keeping trypanosomes alive, it was found without exception that phenyl-arsenoxides containing carboxyl groups had the same lethal effect on normal and tryparsamide-resistant strains of *Trypanosoma rhodesiense*. The trypanocidal activity as shown in Table I was of a relatively low order.

TABLE I.

| Compound. | Dilutions in Millions Lethal in 6 Hours. | |
|---|---|---|
| | Normal Strain. | Resistant Strain. |
| 4-Carboxyphenylarsenoxide . . . | 0·4<br>0·8<br>0·8 | 0·8<br>0·8<br>0·8 |
| 3-Acetamido-4-carboxyphenylarsenoxide . | 0·1 | 0·2 |
| 4-Phenylglycinearsenoxide . . . | 0·8 | 0·8 |
| 2 : 4-Dicarboxyphenylarsenoxide . . | 0·1<br>0·1 | 0·1<br>0·05 |
| 4-Acetamido-3-carboxyphenylarsenoxide . | 0·032 | 0·032 |

We had made these, at first sight, surprising observations when Dr. Hawking told us of his finding that phenylarsenoxide acts equally well on normal and resistant trypanosomes. We were therefore encouraged to extend our experiments to a number of other arsenoxides which we had available. The results are shown in Table II.

From a close perusal of the results in these two tables, we believe that normal trypanosomes can be acted upon by arsenicals in three different ways at least. Moreover, each of these ways is but preliminary to the final chemical action which results in the death of the trypanosome. The compounds shown in Table I all contain carboxyl groups which form neutral sodium salts. These sodium salts are readily water-soluble, and are for the most part present as ions and loth to leave the watery medium. In our opinion, they can only enter the trypanosome in the same way as substances which are very soluble in water, such as glucose and salts. This also is probably the explanation for their relatively low toxicity to the trypanosome.

4-Phenylglycinearsenoxide, $CO_2H . CH_2 . NH . Ph . AsO$, with its acetic acid radicle, falls into this group. This oxide corresponds to arsenophenyl-glycine which was so important in Ehrlich's theories of types or degrees of drug-resistance and to account for which he postulated aceticoceptors in the trypanosome. This oxide also corresponds to phenylglycinearsonic acid, an acid which Yorke, Murgatroyd and Hawking [10] found to be

[14] Cohen, King and Strangeways, *ibid.*, 1931, 3236.

TABLE II.

| Compound. | Dilutions in Millions Lethal in 6 Hours. | | |
|---|---|---|---|
| | Normal Strain. | Resistant Strain. | N/R Ratio. |
| Phenylarsenoxide. PhAsO | 320 | 640 | 0·5 |
| Diglutathionylphenylthioarsinite. $\mathrm{Ph\,.\,As}<^{\mathrm{SG}}_{\mathrm{SG}}$ | 80 | 80 | 1 |
| *p*-Xylylarsenoxide. $\mathrm{Me_2\,.\,Ph\,.\,AsO}$ | 205<br>160 | 205<br>320 | 0·5-1 |
| *p*-Methoxyphenylarsenoxide. MeO . Ph . AsO | 205<br>80 | 205<br>160 | 0·5-1 |
| *p*-Thiomethylphenylarsenoxide. MeS . Ph . AsO | 320<br>320 | 320<br>320 | 1 |
| *p*-Acetophenonearsenoxide. $\mathrm{CH_3\,.\,CO\,.\,Ph\,.\,AsO}$ | 205<br>410 | 102·5<br>205 | 2 |
| *p*-Dimethylanilinoarsenoxide. $\mathrm{NMe_2\,.\,Ph\,.\,AsO}$ | 256 | 128 | 2 |
| *p*-Nitrophenylarsenoxide. $\mathrm{NO_2\,.\,Ph\,.\,AsO}$ | 25·5<br>2<br>64 | 52<br>2<br>128 | 0·5-1 |
| 3-Nitro-4-hydroxyphenylarsenoxide. $\mathrm{(OH)(NO_2)\,.\,Ph\,.\,AsO}$ | 8<br>26 | 4<br>6·4 | 2-4 |
| *p*-Aminophenylarsenoxide. $\mathrm{NH_2\,.\,Ph\,.\,AsO}$ | 4<br>4<br>8 | 1<br>1<br>1 | 4-8 |
| *p*-Methylsulphophenylarsenoxide. $\mathrm{MeSO_2\,.\,Ph\,.\,AsO}$ | 25·6<br>32 | 1·6<br>4 | 8-16 |
| *p*-Hydroxyphenylarsenoxide. OH . Ph . AsO | 51·2<br>160 | 3·2<br>10 | 16 |
| Diglutathionyl-*p*-hydroxyphenylthioarsinite. $\mathrm{OH\,.\,Ph\,.\,As}<^{\mathrm{SG}}_{\mathrm{SG}}$ | 25·6<br>40 | 0·8<br>1·25 | 32 |
| Phenyl-*pp′*-diarsenoxide. AsO . Ph . AsO | 32 | 1 | 32 |
| *p*-Acetanilidearsenoxide. NHAc . Ph . AsO | 51·2 | 1·6 | 32 |
| Diglutathionyltryparsamidethioarsenite. $\mathrm{CONH_2\,.\,CH_2\,.\,NH\,.\,Ph\,.\,As}<^{\mathrm{SG}}_{\mathrm{SG}}$ | 12·8 | 0·4 | 32 |
| Diglutathionyl-2-hydroxy-4-acetamido-phenylthioarsinite. $\mathrm{(NHAc)(OH)Ph\,.\,As}<^{\mathrm{SG}}_{\mathrm{SG}}$ | 12·8<br>25·6 | 0·2<br>0·8 | 32-64 |
| Dicysteinylbenzamide-*p*-thioarsinite. $\mathrm{CONH_2\,.\,Ph\,.\,As}<^{\mathrm{SC}}_{\mathrm{SC}}$ | 25·6<br>51·2 | 0·8<br>0·6 | 32 |
| Benzamide-*p*-arsenoxide. $\mathrm{CONH_2\,.\,Ph\,.\,AsO}$ | 51·2<br>51·2 | 0·8<br>0·8 | 64 |

anomalous. The fact that this oxide with free carboxyl group falls among these other oxides all with free carboxyl groups suggest that the critical factor in the behaviour of these phenylglycine derivatives is the carboxyl group and that postulation of aceticoceptors is redundant. The observed phenomena are more readily interpreted on the basis of formation of water-soluble salts through the carboxyl groups and the distribution of this group of drugs containing carboxyl groups through the water phase as ions.

The distribution of the substances in Table II in or on the trypanosome is quite different from that of the ionised substances, and they act in at least two other ways. At one end of the table such substances as phenyl-arsenoxide and xylylarsenoxide are very active in high dilutions *in vitro*

on trypanosomes, on both normal and resistant strains alike. In chemical structure, apart from the arsenoxide group, they are devoid of markedly polar or hydrophilic groups. A few years ago Kligler and Olitzki [15] showed that in *Trypanosoma evansi* 60 % of the trypanosome was lipoidal in nature. We therefore venture to suggest that phenyl- and xylyl-arsenoxides are taken up at a lipoidal-water interface in such a way that the phenyl or xylyl group is in the lipoid and the arsenoxide group is at the water interface. By such a mechanism they seem to be transported in a very facile manner, since their action is so very pronounced, to a site where the arsenoxide can exert its lethal chemical action. Since these substances act equally well on normal and resistant strains, they must act in a different way and be differently distributed from the seven substances, for example, at the bottom of the second table. These latter substances belong to the group of arsenicals in which drug-fastness was first found by Ehrlich, and they act on resistant strains only at concentrations which are between 32 and 64 times stronger than those which still act on the normal forms. These arsenoxides we suppose must be taken up by the surface or some structure in the normal trypanosome in the same way as the group of dyes represented by the acridines or oxazines. They are, in fact, substantive for the same type of structure. Of what this structure is composed in the trypanosome we know very little except that it is presumably of polar nature and not capable of adsorbing dyes of the congo-red or triphenylmethane types. We picture this group of arsenicals as being waylaid by adsorption on polar surfaces in normal trypanosomes *en route* to their site of action, since the concentration at which benzamidearsenoxide acts, at one extreme of the table, is so much greater than that at which phenylarsenoxide acts at the other end of the table.

Does the arsenoxide group take part in the primary fixation or are the other substituents solely responsible ? To try and decide this question, phenyl-*pp′*-diarsenoxide was synthesised, and when tested on normal and resistant strains, was found to belong to the atoxyl group of arsenicals, the N/R ratio being 32. This observation leads support to the view that in the primary fixation of this type of aromatic arsenical, both ends of the molecule are involved, and the molecule as a whole lies flat on the adsorbing surface.

In conclusion, it is tempting to try and complete the picture of the mode of action of arsenicals. The three types of distribution of aromatic arsenoxides in or on the trypanosome discussed above are merely the primary phase in the action. The final mechanism is a chemical one in which the highly reactive arsenoxide group—the toxophoric group—combines with some essential cell constituent on which the life of the trypanosome depends. With a complete knowledge of the structure and of all the reactions going on in the trypanosome-cell and of the enzymes involved, it should be possible to be certain of the final mechanism of action of an arsenical, but this state of knowledge is impossible to attain without the aid of Maxwell's sorting demons. Arsenoxides possess an intense affinity for SH groups and a water-insoluble arsenoxide dissolves readily when dusted on to the surface of a solution of glutathione. It was shown by Lohmann [16] that glutathione is a specific co-enzyme for glyoxalase, an enzyme of wide distribution. If, therefore, the life of a trypanosome is dependent on glyoxalase, then this enzyme cannot function in presence of arsenoxide and the trypanosome must perish. Alternatively, there are other more important enzymes containing SH in their protein structure whose activity is dependent on the maintenance of SH in the reduced form,[17] a condition incompatible with the presence of arsenoxides.

[15] Kligler and Olitzki, *Ann. Trop. Med. Parasit.*, 1936, **30**, 287.

[16] Lohmann, *Biochem. Z.*, 1932, **254**, 332.

[17] Barron and Singer *Science*, 1943, **97**, 356 ; Hellerman, *Cold Spring Harbor Symp.*, 1939, **7**, 165 ; Bernheim and Bernheim, *ibid.*, 174 ; Hellerman, Chinard and Deitz, *J. Biol. Chem.*, 1943, **147**, 443.

## *GENERAL DISCUSSION*

**Dr. E. M. Lourie** (*Liverpool*) said : Dr. King has drawn attention to Ehrlich's finding that trypanosomes made resistant to atoxyl and certain other arsenicals, as a result of treatment by subcurative doses of these drugs, become resistant also to non-arsenical acridine compounds. Ehrlich showed also that, conversely, trypanosomes treated by acridines are liable to acquire, coincidentally, a resistance to arsenicals. This phenomenon introduces a present-day bogey which it is necessary to enquire into, and which arises in the following way.

Mepacrine is being increasingly used for malaria in the place of quinine. Now, mepacrine is an acridine compound, and the question therefore arises whether the unrestricted use of this drug in the sleeping-sickness areas of Africa will not result in the production of strains of trypanosome resistant to arsenicals. This would be a serious matter, since arsenical compounds are, at present, the only known effective remedies for the latest stages of sleeping-sickness. Dr. Collier and I have, however, investigated the matter experimentally, and we find that very intensive treatment of trypanosomes by mepacrine, both *in vivo* and *in vitro*, does not produce a strain of parasite resistant to arsenical compounds. We conclude, therefore, that the danger of giving rise to such strains in Africa, by the increased use of mepacrine, may be dismissed as negligible.

**Dr. M. A. Phillips** (*London*) asked whether anything was known of the relationship, if any, of ease of de-arsenication to trypanocidal action. From the chemical point of view, de-arsenication was particularly evident in compounds containing an hydroxy group *ortho* to the arsenic atom.

**Dr. King** replied that he knew of no evidence in support.

**Dr. H. Hurst** (*Cambridge*) (*communicated*) : The experimental evidence provided by Dr. King lends further support to the theory that the cell membrane may be regarded as a visco-elastic lipo-protein mosaic structure in which primary drug fixation regulates the accessibility of the drugs to the relatively internal enzyme substrate. The work of Kligler and Olitzki indicates that the cell membrane of the parasite is relatively rich in lipoid material. That this lipoid material does not constitute a *continuous* layer or layers parallel to the cell surface is shown by the fact that drug activity cannot be correlated with the simple energy requirements of differential solubility or adsorption. The activity of phenylarsenoxide is reduced by the introduction of two oil-solubilising methylene groups into the molecule to form *p*-xylylarsenoxide, a change in molecular structure which also involves an increase in the free energy of adsorption of the molecule at an oil/water interface.

The fall in activity produced by the introduction of an additional arsenoxide group into the phenylarsenoxide molecule suggests that the enzyme substrate at which the ultimate toxic drug interaction takes place is not directly accessible to the external drug phase, since if this were the case, the availability of the arsenoxide toxophoric group would be increased by the change in the drug molecule.

The above evidence suggests that drug access is influenced both by the hydrophobic and hydrophilic portions of the molecule. The nature of the interaction of the drug molecules with the components of the cell wall may be deduced from the following considerations :

The velocity of spreading of capillary active substances at an air/water interface is very high, and may be of the order of 20 cm. per second. The *driving force* which influences this high rate of spreading is due to the high two-dimensional concentration gradient along the interface ; three-dimensional diffusion in the underlying bulk water phase is of a very low order, since there is no selective concentration of the capillary active molecules in the bulk phase. At an oil/water interface, two-dimensional

diffusion may be modified by the interaction of the orientated hydrophobic portion or portions of the molecules with the adjacent oil phase. In this system selectivity factors may be introduced by changes in the gross physical properties of the bulk oil and water phases, such as alteration in viscosity, or by the presence of fixed structural receptor groups which may " anchor " the capillary active molecules at the interface.

If the molecule of phenylarsenoxide is selected as a " standard," certain general conclusions may be deduced from the data summarised in Tables I and II.

(1) Trypanocidal activity is at a maximum in capillary active drugs in which the molecule contains a single arsenoxide polar group (phenylarsenoxide ; *p*-xylylarsenoxide).

(2) The introduction of additional single polar groups into the " standard " molecule results in a loss in activity (phenyl-*pp'*-diarsenoxide ; *p*-hydroxyphenolarsenoxide ; *p*-nitrophenylarsenoxide ; *p*-aminophenylarsenide).

(3) With further increase in the number of polar groups, there is a corresponding fall in drug activity (2 : 4-dicarboxyphenylarsenoxide ; 4-carboxyphenylarsenoxide ; 4-acetamido-3-carboxyphenylarsenoxide).

The pronounced activity of dilute aqueous solutions of phenylarsenoxide and *p*-xylylarsenoxide suggests that primary drug fixation involves selective adsorption of the drug molecules at lipo-protein interfaces in the bounding cell wall of the parasite. Rapid access to the internal enzyme substrate can only be achieved when these interfaces constitute a series of channels or pathways which communicate more or less directly with the internal biophase. The drug concentration gradient across the cell wall will consist of a series of localised two-dimensional concentration gradients of high magnitude, and drug mobility will be influenced by polar and non-polar interaction of the reactants in the system. The magnitude of the changes in activity which occur when additional polar groups are introduced into the drug molecule may be attributed partly to the decrease in capillary activity, and partly to a relatively non-specific multipolar interaction between the polar portions of the drug molecules and the visco-elastic protein components at the Gibbs' layer. These factors account for the feeble trypanocidal activity of compounds such as 4-carboxyphenylarsenoxide, where the presence of water-solubilising groups will minimise adsorption at the Gibbs' layer. Drug access will mainly involve a slow three-dimensional diffusion across the bulk protein channels in the cell membrane framework, and activity will depend on the presence of relatively high drug concentrations in the bulk external drug phase.

A complicating factor in the correlation of molecular structure with trypanocidal activity is introduced by the possibility that the functional susceptibility of the parasite is influenced by the carrier activity of the drugs at the site of primary drug fixation. Further evidence in support of this might be provided from experiments on mixed drug systems.

It is interesting to note that the insecticidal activity of ethyl alcohol or phenol falls with the introduction of additional hydroxyl groups into the drug molecules ; ethylene glycol and resorcinol are only feebly active. Analogous results have been obtained for drug hæmolytic activity on mammalian erythrocytes.[18]

[18] Jacobs, Glassman and Parpart, *J. Cell. Comp. Physiol.*, 1935, **7**, 197.

# PRINCIPLES OF INSECTICIDAL ACTION AS A GUIDE TO DRUG REACTIVITY-PHASE DISTRIBUTION RELATIONSHIPS.

By H. Hurst.

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Considerable experimental evidence [1] has accumulated which shows that the biological activity of a drug may depend on molecular interaction with the biological system involving (i) differential solubility in the cell lipoids [2] (Overton-Meyer), (ii) adsorption at cell interfaces [3] (Traube), or more specifically (iii) polar interaction, van der Waals' non-polar interaction, and specific stereochemical relationships of the reactants in the system.[4]

The majority of the pharmacological systems which have been used in quantitative measurements of drug action are of the type in which an aqueous carrier medium serves to bring the drugs into contact with the biological system. Drug reactivity is then measured by the determination of the molar concentration of the drug which results in the production of a specific response in the biological system. It has generally been assumed that the main function of a carrier medium is to bring the drug into contact with the biological system. It has also been assumed that, where drug access to the site of action is limited by a diffusion process through an intermediary biophase or membrane, the rate of drug access is proportional to the difference between drug concentration in the external carrier medium or "source," and in the site of action or "sink." This application of Fick's Law to quantitative interpretations of drug reactivity has formed a basis which is fundamental to all permeability studies.

In the present paper the validity of these fundamental assumptions will be considered in the light of experimental evidence which has resulted during the course of a recent investigation on the mode of insecticidal action.

## Drug Concentration-Biological Activity Relationships.

When immersed in pure kerosene or ethyl alcohol, mature *Calliphora erythrocephala* blowfly larvæ show no marked symptoms of paralysis even after an hour or longer. Mixtures of ethyl alcohol and kerosene are extremely toxic, and the insects are immobilised within a few seconds. The permeability of the cuticle is so high in the presence of kerosene that rapid access of alcohol into the internal body fluids of the insect results in an

[1] (*a*) Fühner, *Arch. exp. Path. Pharmak.*, 1904, **51**, 1, and 1904, **52**, 69. (*b*) *Biochem. Z.*, 1921, **115**, 235. (*c*) Warburg, *Biochem. Z.*, 1921, **119**, 134. (*d*) Winterstein, *Die Narkose*, 2nd ed., Berlin, 1926. (*e*) Tilley and Schaffer, *J. Bact.*, 1926, **12**, 303. (*f*) Clark, *The Mode of Action of Drugs on Cells*, 1933, London. (*g*) Clark, *Trans. Faraday Soc.*, 1937, 1057 (General Discussion on the Properties and Functions of Membranes, Natural and Artificial). (*h*) Meyer, *ibid.*, 1062. (*i*) Meyer and Hemmi, *Biochem. Z.*, 1935, **277**, 39. (*j*) Ferguson, *Proc. Roy. Soc.*, *B*, 1939, **127**, 387. (*k*) Davson and Danielli, *The Permeability of Natural Membranes*, 1943, Cambridge.

[2] (*a*) Overton, *Jahr. Wiss. Bot.*, 1900, **34**, 669; (*b*) *Studien über Narkose*, 1901, Jena.

[3] (*a*) Traube, *Pflügers Arch.*, 1904, **105**, 541; (*b*) *ibid.*, 1908, **123**, 419; (*c*) *Verhandl. deutsch. Physik. Ges.*, 1909, **10**, 800.

[4] (*a*) Schulman and Rideal, *Proc. Roy. Soc.*, *B*, 1937, **122**, 29. (*b*) Schulman and Stenhagen, *ibid.*, 1938, **126**, 356. (*c*) Schulman and Rideal, *Nature*, 1939, **144**, 100.

increase in volume of about 50 % within three minutes. This stage is followed by the bursting of the insect when the internal pressure developed becomes excessive. These effects are produced with great rapidity by drug mixtures containing 20 to 80 % alcohol. On either side of this range equitoxic effects may be produced, showing that drug concentration in the carrier medium is not necessarily a limiting factor in biological activity when an external insecticidal mixture is brought into contact with an insect. Where the concentration of alcohol is low, biological activity increases with increase in drug concentration. But where the concentration of ethyl alcohol is high, toxicity decreases with increase in drug concentration, since within this range cuticle permeability decreases to a minimum as drug concentration increases to a maximum.

The relatively enormous increase in cuticle permeability which is induced by kerosene illustrates the influence of a diffusion factor in drug access. The importance of carrier activity is shown by the fact that ethyl alcohol-water mixtures are relatively non-toxic for all dilution ranges.

Similar effects of "induced drug access" by fat solvents such as kerosene are shown by other primary alcohols, fatty acids, ketones, amines, and phenols.[5] In these systems, the biological activities of given drug concentrations in kerosene are logarithmically greater than the corresponding biological activities in aqueous carrier media. From this evidence, the following conclusions may be drawn:

(1) All components of a drug mixture may contribute towards gross biological activity.

(2) The carrier in a drug mixture may participate functionally in the biological system so as to modify the "functional susceptibility" of the biological system.

(3) Fick's Law is invalid as a general basis for drug concentration-biological activity relationships. Drug access may increase, remain constant, or decrease with increase in drug concentration, according to the associated changes in carrier activity.

## Drug-Biological System Phase Distribution Relationships.

An insight into the nature of the association of drugs with the intermediary biophases in a biological system which link the site of drug application with the site of drug action is provided from an examination of the insecticidal action of members of the homologous series of primary alcohols and fatty acids.[6] In these series of drugs corresponding homologues differ only in the polar portions of the molecules.

The simplest method of external drug application consists in immersing the test insects in relatively large proportions of pure drug. Since the external drug phase is large in relation to the biological system, the disturbing influences of drug depletion in the external phase, and drug distribution between the carrier medium and the insect, involving possible solubility or adsorption factors, are avoided. In the homologous series of normal primary alcohols and fatty acids, the relative molar concentrations of the pure drugs decrease with increase in chain length owing to an increase in molecular volume. The total decrease in the molar concentration in each series is roughly fourfold, ascending the series from $C_1$ to $C_8$. The absolute molar concentrations of corresponding members in both series are approximately equal.

The bulk internal biophase of an insect is essentially aqueous. When a solution of a drug in a non-aqueous carrier is injected into the hæmolymph, complications which might arise owing to the possible influence on biological activity of drug distribution between the mutually immiscible hæmolymph/carrier phases may be avoided by the injection of aqueous drug solutions.

[5] Hurst, *Nature*, 1940, **145**, 462. [6] *Ibid.*, 1943, **152**, 292.

The relative external and internal biological activities of members of the homologous series of normal primary alcohols and fatty acids are shown in the graphs (Fig. 1 (*a*), (*b*)). Each point on the curves represents the average of 10 determinations of biological activity on insects selected from a uniform batch (mature *Phormia terrænovæ* blowfly larvæ). The drugs were administered externally by immersing the test insects in 10 c.c. samples of each member of the drug series. Biological activity was measured on a time-action basis when the drugs were applied externally. Survival time gave the most clearly defined criterion of activity under these conditions (Fig. 1 (*a*)). Paralysis was selected as an index of internal biological activity when the drugs were injected directly into the hæmolymph; owing to gradual leakage of drug and internal body fluids from the site of injection, death was a less reliable criterion of biological activity than paralysis. Comparison between external and internal drug reactivities may be made on the assumption that external biological activity is inversely proportional to survival time, and internal biological activity inversely proportional to the isoactive molar concentrations in the dosages injected, which were roughly 25 mg./100 mg. body weight. The reciprocals of the threshold molar concentrations which induce paralysis are expressed as arbitrary numerical indices of molecular activity (Fig. 1 (*b*)).

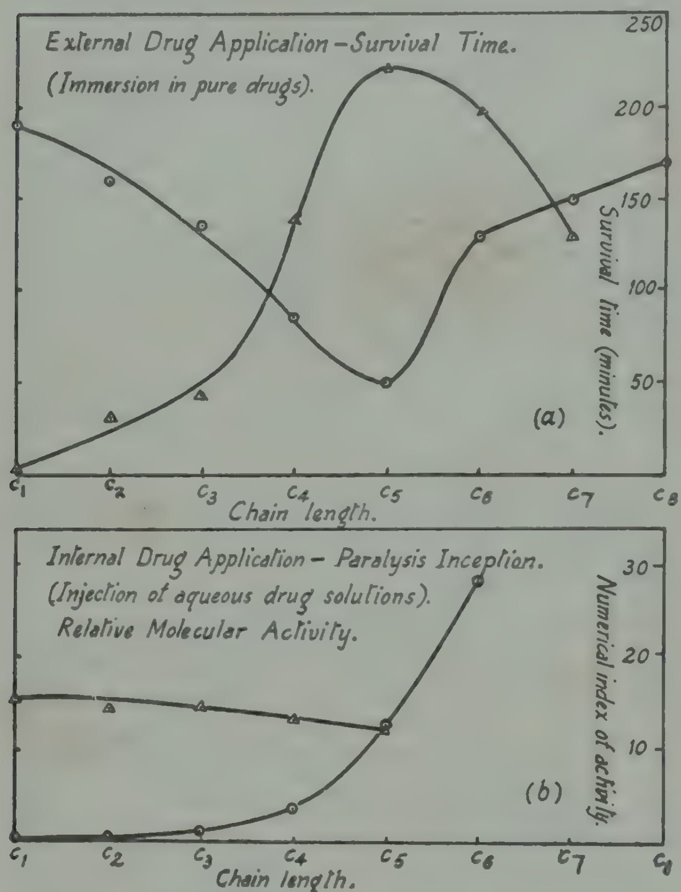


FIG. 1—The relative external and internal biological activities of members of the homologous series of normal primary alcohols and fatty acids (Test Insect, *Phormia terraenovae*).

⊙ Alcohols. △ Fatty acids.

Analysis of the data summarised in the graphs shows that drug access cannot be correlated with simple drug-cuticle phase distribution relationships. Within the range $C_1$ to $C_5$ the penetration of the alcohols increases with chain length and reaches a maximum at $C_5$. Within a corresponding range, there is a relatively enormous decrease in the rate of penetration of the fatty acids; drug access is at a minimum at a chain length of $C_5$. This divergence in drug access as both series of drugs are ascended from $C_1$ to $C_5$ changes to a convergence as the series are ascended further from $C_5$ to $C_7$. At $C_7$ survival times are of a similar order in both series. ($C_8$ in the fatty acid series is solid at normal room temperatures, and is therefore unsuitable for comparison with the corresponding liquid alcohol homologue.)

Difficulties arise when an attempt is made to correlate the relative external biological activities of the drugs with the internal biological activities. For systems in which the drugs are applied internally, there is a rough proportionality in the alcohol series between molecular activity and chain length within the restricted range $C_1$ to $C_5$. In the fatty acid series, internal application shows that the *isoactive molar concentrations remain relatively constant* as the series is ascended from $C_1$ to $C_5$. Increase in chain length produces no increase in biological activity which is comparable with the exponential rate of change shown by the alcohols within this range. Where the chain length is short ($C_1$) the molecular activity of the fatty acids (formic acid) is roughly about 80 times that of the alcohol homologue (methyl alcohol). The dominant influence of chain length in both series is shown at $C_5$ when valeric acid is isoactive with amyl alcohol. In both series the *intensity* of narcosis produced by internal drug application decreases as the chain length exceeds $C_5$. This change is most pronounced in the fatty acid series, and saturated aqueous drug solutions produce only incomplete paralysis.

From this evidence the following conclusions may be drawn:

(1) Where diffusion through an intermediary biophase which links the site of drug application with the site of drug interaction in the biological system is a limiting factor in drug access, relative biological activity measured at the site of drug application may differ from relative drug activity at the ultimate site of action. Drug reactivity may increase, remain relatively constant, or decrease as a homologous series of drugs is ascended, according to the mode and the site of application.

(2) Quantitative measurements of drug reactivity have generally been based on comparison of the molar concentrations which produce equi-toxic or equi-narcotic effects on selected biological test systems. The mode of application of a drug is generally severely restricted by the biological component of the pharmacological system. For example, systems such as erythrocytes, protozoa, bacteria, or isolated preparations of heart, muscle, nerve, and other tissues are usually examined when in contact with physiological media of which water is a major component. The ranges of drugs and drug concentrations are limited by physico-chemical factors, such as solubility, and the aqueous carrier medium must be carefully selected to maintain a condition of physiological balance with the biological system during the period of experimental observation. In systems of this type, correlation of drug reactivity with solubility in the cell lipoids (Overton-Meyer) or with adsorption at cell interfaces (Traube) may lead to ambiguity, since in homologous series, physical properties such as differential oil/water solubility, capillary activity, viscosity, or vapour pressure *change uniformly in the same direction* as the series are ascended. An exponential relation between molecular drug activity and length of hydrocarbon chain may simply express the tendency of the hydrocarbon portions of the drug molecules to escape from the aqueous carrier medium into the lipoid centres of the biological system. Since these lipoids are invariably associated with other relatively hydrophilic substances (*e.g.* proteins) to form complex systems of interfaces, it is difficult to exclude the possibility of adsorption as a factor in drug-biological system phase distribution relationships where the drugs are capillary active.

This complicating influence of phase distribution between a carrier medium and the biological component of a pharmacological system disappears when the biological system is brought into contact with pure drugs. Biological activity is now chiefly influenced by molecular interaction in the actual biophases of the biological system. By the use of relatively resistant test organisms, such as blowfly larvæ, the influence of both polar and non-polar portions of the drug molecules is shown clearly by the gross divergence between the relative biological activities of the alcohols and fatty acids when corresponding homologues are compared. The

results recorded in Fig. 1 show that drug reactivity cannot be attributed solely to simple distribution between immiscible phases based on differential solubility or adsorption factors.

## Mechanism of Drug Access.

The experimental evidence which has been provided shows that the measurement of the biological activity of a drug involves a precise definition of the nature and site of drug interaction. When drugs are applied externally to insects, the *primary* site of drug interaction, irrespective of biological response, is at the external surface of the cuticle. *Secondary* interactions may occur at specific receptors or loci in the intermediary chain of biophases (i) bulk cuticle framework, (ii) hæmolymph and associated tissues.

**Nature of Cuticle Surface.**—When blowfly larvæ are suspended at an air/water interface, a monomolecular film spreads outwards along the interface. This surface film gels reversible at low surface pressures (2-4 dynes/cm.), and becomes coherent and rigid when tannic acid is injected into the underlying water phase. This indicates the presence of proteins at the cuticle surface, since similar effects are shown with long chain amine or protein monolayers. The "tanning" of these films is due to the multiple-point interaction of the polar groups in the tannic acid molecule with the ionised amine groups in the surface film.[18]

The presence of lipoid components at the cuticle surface is shown by the narcotic action of fat solvents. Blowfly larvæ become rapidly paralysed when immersed in the lower members ($C_6$ to $C_8$) of the homologous series of paraffins. Narcosis occurs within 1 to 2 minutes, even when the entrance of the drugs into the tracheæ is precluded by blockage of the spiracles. Since paraffins are insoluble in proteins, drug access must take place through lipoid loci at the cuticle surface. From this evidence it may be concluded that the surface of the cuticle in blowfly larvæ is not homogeneous but composite, and consists of heterogeneous protein and lipoid associations or "patches" suggestive of a mosaic ultrastructure.

**Bulk Cuticle Framework.**—There are two primary layers in a typical insect cuticle, an outer "lipoid" layer, or epicuticle, and an inner chitin-protein layer.[7] The epicuticle is secreted as a thin hydrophilic membrane by the epidermal cells. It is later "tanned" and impregnated with lipoid material, which by a process of condensation and polymerisation becomes relatively resistant to the disruptive action of fat solvents and strong mineral acids.[8] The inner layer of the cuticle consists of a chitin framework which is closely associated with the protein components. These are relatively water-soluble in blowfly larvæ.[9] The bulk cuticle phase is permeated by a fatty secretion of the epidermal cells. This secretion passes through the cuticle along specialised ducts to the outer epicuticle; there is probably a more general transmission of lipoid secretion through the "pore canals" which form a network in the inner chitin-protein layer but do not pass through the epicuticle framework.

It is clear from this brief description of the gross morphology that the cuticle consists of a complex system of heterogeneous phases, and correlation of biological activity of drug systems present as an external phase with cuticle permeability involves a specific definition of the limiting factors in drug-cuticle interaction.

**Action of Fat Solvents.**—Early in the present account it was shown that fat solvents such as kerosene exert a marked synergistic action on

[7] Kühnelt, *Zool. Jahrb. Abt. Anat.*, 1928, **50**, 219.

[8] (*a*) Wigglesworth, *Quart. J. Micr. Sci.*, 1933, **76**, 270; (*b*) *The Principles of Insect Physiology*, 1939, London. (*c*) Pryor, *Proc. Roy. Soc.*, *B*, 1940, **128**, 393.

[9] (*a*) Fraenkel and Rudall, *ibid.*, **129**, 1. (*b*) Trim, *Biochem. J.*, 1941, **35**, 1088.

the rate of transmission of ethyl alcohol through the cuticle of blowfly larvæ. Not only does the insect swell owing to rapid diffusion of the alcohol into the internal body fluids, but a cloudy swirling zone appears immediately outside the cuticle owing to the displacement of kerosene from the drug mixture by water which diffuses outwards from the cuticle.[5] Similar effects have been described for other insects. Water also passes outwards from the cuticle when the insects are immersed in oils alone; owing to insolubility in the oil phase, the water assumes the form of minute droplets attached to the surface of the cuticle.[10]

Induced access of drugs owing to carrier activity does not depend primarily on vital transport through the bulk cuticle framework; fat solvents such as kerosene induce drug access through the cuticles of dead blowfly larvæ. Analogous effects have also been obtained in synthetic systems in which a drug phase is separated from an aqueous phase by an isolated cuticle "membrane," or by a discrete component layer of the cuticle. The permeability of all component layers of the cuticle is increased by kerosene. The degree of induced drug penetration is most marked through the extreme outer layer, or epicuticle,[5] in which the preponderance of lipoid constituents has been demonstrated histologically.

The association of fat solvents with the cuticle framework is primarily physical, and the changes in permeability which are induced are readily reversible. For example, *Calliphora* larvæ will swell rapidly in ethyl alcohol-kerosene mixtures, but further access of alcohol ceases when the insects are transferred to pure ethyl alcohol owing to "wash-out" of kerosene from the cuticle framework. Similar effects are shown with isolated cuticle preparations. This shows clearly the reversible nature of the functional changes in cuticle permeability which are induced by the association of the carrier in a drug mixture with the cuticle framework.

After prolonged immersion in fat solvents, an irreversible increase in cuticle permeability takes place. This is shown by (i) an increase in the original rate of water loss by evaporation through the cuticle, and (ii) by a marked increase in the rate of access of pure drugs such as methyl or ethyl alcohol. After this treatment, the rate of drug access is still further increased by oil carriers such as kerosene, showing that only a proportion of the lipoid components are dispersed or removed by fat solvent action. This provides striking evidence that drug access does not depend primarily on solubility in the lipoids, since removal of lipoids from the cuticle would hardly facilitate drug access if this were the case. The main site of carrier activity is in the outer layers of the outer layer of the cuticle. When the epicuticle is removed or mechanically damaged, the insect loses its capacity for the conservation of water and rapidly becomes desiccated, showing that the inner chitin-protein layer is relatively permeable to water. When the epicuticle is removed carrier activity in the residual cuticle layers is relatively slight.

The physical nature of the oil carrier has a marked influence on carrier activity. In the series hexane $\rightarrow$ octane $\rightarrow$ decane $\rightarrow$ dodecane (kerosene range) $\rightarrow$ heavy oil (medicinal paraffin range) carrier activity decreases with increase in viscosity of the carrier. These factors are relatively independent of carrier toxicity. For example, narcotic activity of the homologous series of paraffins to blowfly larvæ shows a sharp "cut-off" as the series is ascended in the range $C_8 \rightarrow C_{10}$; dodecane ($C_{12}$) is relatively non-toxic. Within the range $C_6 \rightarrow C_{12}$ carrier activity is very pronounced, but the penetration of ethyl alcohol through the cuticle is only moderately increased by relatively viscous oils such as "thick Nujol," or medicinal paraffin.

[10] Wigglesworth, *Bull. Ent. Res.*, 1942, **33**, 205.

## Physico-chemical Changes in Cuticle Framework produced by the Action of Fat Solvents.

From the experimental evidence which has been provided the changes in functional cuticle permeability which are produced by the association of fat solvent carriers with the cuticle framework may be attributed to :

(i) A van der Waals' interaction between the fat solvent molecules and the lipoids of the cuticle, involving a reduction in mutual cohesion between the lipophilic elements or chains, and resulting in the production of a relatively open three-dimensional chemically linked network which encloses the more " labile " lipoid components in which the solvent molecules participate functionally. In this way the fat solvent increases the free volume of the lipoid phase, and may also act as a " bridging " medium which links discrete lipophilic patches in the bulk cuticle phase. Since both protein and lipoid components are enclosed in an elastic lattice framework, molecular alterations in the lipoid phase will probably induce associated changes in molecular orientation in the protein phase. The decrease in carrier activity which takes place with increase in carrier viscosity suggests that the bulk lipoid phase is normally of high " functional viscosity." The term " functional viscosity " is not strictly comparable with Newtonian viscosity in a homogeneous liquid, since the protein and lipoid phases of the cuticle are heterogeneous visco-elastic systems comprising chemically-linked networks which enclose the more " mobile " components. " Functional viscosity " gives a measure of the " yield value " of the lipoid phase to drug diffusion pressure.

(ii) A secondary irreversible increase in the flexibility of the lipophilic lattice elements owing to a rupture of the cross-linkages which normally regulate the stability and elasticity of the lipophilic chains. The elastic restoring forces of the lattice framework oppose the dispersive or disruptive action of the fat solvent. The irreversible increase in permeability, which probably involves an increase in free volume in the bulk lipoid phase, is due to the removal of " labile " bonding components by fat solvent action, resulting in increased flexibility or mobility of the residual lattice chains. This would account for the increase in the mobility of water molecules especially in the outer layers or epicuticle, and also explains the increase in the permeability of the cuticle to drugs such as fat soluble alcohols when a proportion of the lipoids of the cuticle have been removed by prolonged fat solvent action.

## Action of "Inert" Powders or Dusts.

Additional evidence which supports the suggestion that the labile cuticle components act as a " bonding " phase which regulates the cohesion of the chemically-linked cuticle lattice elements is provided by the insecticidal action of " inert " powders or dusts.

Finely divided powders such as quartz or charcoal readily adsorb the relatively labile lipoid or lipo-protein components from the surface of the epicuticle. This process is non-specific, and depends on the adsorptive and storage capacities of the powder for the capillary active cuticle components, and also on the capacity of the epicuticle/powder interface for the transmission of these components. When the rate of removal of lipoid by adsorption displacement exceeds its rate of replenishment by transmission from the epidermal cells to the epicuticle, irreversible secondary changes are produced in the epicuticle framework. This suggests that the labile components of the epicuticle constitute a bridging or bonding phase which links the flexible lipophilic elements of the epicuticle framework. Depletion of this labile " bonding " phase results in a reduction in lateral cohesion of the lattice elements, and this permits an increase in the mobility of water molecules in the bulk epicuticle phase. Once this

stage is reached, the insect is unable to control the rate of transmission of water through the cuticle, even when the external powder phase is removed, and rapid water loss soon results in death from desiccation.

In the normal insect, variation in the rate of secretion of the "bonding" phase by the epidermal cells may result in corresponding changes in free volume and functional viscosity of the bulk epicuticle lipoid phase, thus providing a very sensitive regulatory mechanism for the control of water loss. "Inert" dusts also increase the rate of water loss through the cuticle of dead insects, although the "latent" period between dust application and eventual desiccation is relatively small owing to the absence of secretory activity of the epidermal cells. This is of interest, since in the living insect the regulatory action of the spiracles is a disturbing influence in the assessment of dust/cuticle water loss relationships. Analogous effects, which have been obtained in synthetic systems using artificial membranes as models of insect cuticle, have provided a very useful basis for predicting the toxicity of insecticidal dusts.

## Carrier and Dispersant Action in Mixed Drug Systems.

Extending these concepts of carrier action, it is seen that a drug may either induce its own access through the cuticle or exert a carrier action in mixed drug systems which is similar to that produced by fat solvents. For example, the survival time of *Calliphora erythrocephala* larvæ in ethyl alcohol-octyl alcohol mixtures is shorter than the respective survival times in the pure drug components. This test insect is more susceptible than *Phormia terrænovæ* larvæ. Typical data showing the change in survival time with change in the relative proportions of the components in ethyl alcohol-octyl alcohol mixtures are shown in Table I. The data in the table illustrate an example of *drug synergism* in which selective access of one component (ethyl alcohol) into the internal biophases of the biological system is induced by selective carrier action of another component (octyl alcohol) at a biophase, the cuticle, which is remote from the ultimate site of drug interaction. The action of octyl alcohol is similar to that of kerosene, but since the octyl alcohol itself is active, a factor of *carrier toxicity* is also involved in addition to *carrier activity*.

TABLE I.—BIOLOGICAL ACTIVITY OF ETHYL ALCOHOL-OCTYL ALCOHOL MIXTURES.

| Drug System % Ethyl Alcohol. | Survival Time (mins.) (*Calliphora* larvae). |
|---|---|
| 100 | 97 |
| 75 | 16 |
| 50 | 1·5 |
| 25 | 3 |
| 0 | 7·5 |

TABLE II.—BIOLOGICAL ACTIVITY OF AMYL ALCOHOL-CETYL ALCOHOL MIXTURES.

| Drug System % Amyl Alcohol. | Survival Time (mins.) (*Calliphora* larvae). |
|---|---|
| 100 | 5·5 |
| 99 | 6·5 |
| 97 | 9·5 |
| 95 | 19·5 |

Comparison of carrier activities in various mixed drug systems shows that in the homologous series of alcohols, carrier activity increases to a maximum and then decreases as the series is ascended and capillary activity becomes pronounced. In fact, strongly capillary active alcohols such as cetyl alcohol exert "negative carrier activity," or *antagonism* on the rate of access of drugs of relatively low capillary activity (Table II).

It is seen from the data in the table that relatively small proportions of cetyl alcohol are effective in reducing the biological activity of the major component of the drug mixture (amyl alcohol).

In the homologous series of fatty acids, carrier activity is less marked than in the alcohol series and becomes pronounced at a later stage ($C_6$ to $C_7$) than in the alcohols ($C_4$ to $C_5$).

## Drug-cuticle Phase Distribution Relationships.

The experimental evidence which has been here provided may be used as a basis for the interpretation of more specific factors in drug access. It is clear that the cuticle framework cannot be regarded as a homogeneous phase, but is more analogous to a composite visco-elastic system of liquid/liquid interfaces, comprising a hydrophilic protein gel phase which is associated with a hydrophilic lipoid phase of high " functional viscosity."

Drug penetration through the bulk cuticle framework may involve:

(1) Diffusion through the bulk protein phase.

(2) Diffusion through the bulk lipoid phase.

(3) Free diffusion through the pores or canals in the bulk cuticle framework.

(4) Free diffusion through a bulk cuticle phase in which the lipoid and protein components are rendered mutually miscible, or functionally homogeneous by the dispersant action of the drug components.

(5) Two-dimensional diffusion along the Gibbs' layer, or internal network of liquid/liquid interfaces.

The experimental evidence does not support drug access through the bulk protein or lipoid phases. From differential solubility considerations alone, the rate of change in drug access should proceed uniformly and in the same direction in both homologous series of alcohols and fatty acids as the series are ascended. Similarly, any attempt to correlate drug penetration with adsorption at liquid interfaces of the oil/water type is precluded, since the work of adsorption of alcohols and fatty acids at an oil/water interface increases uniformly in both series as surface activity increases.

Structurally, the cuticle framework contains an extensive system of " pore canals " which extend from the inner epidermal cells to the inner surface of the epicuticle.[8a, b, 10, 11] That molecular " sieve action " is not a limiting factor in drug access is shown by the following evidence:

(i) The main site of induced drug access is in the outer epicuticle layer, which does not contain macroscopic pore canals.[8b]

(ii) Molecular volume increases in both series of alcohols and fatty acids as the series are ascended. Drug access may either increase or decrease in both series (Fig. 1 (*a*)).

The possibility also exists that interaction of the external drug mixture with the heterogeneous lipoid and protein components of the cuticle induces mutual dispersion or " emulsification " of these components so that drug access is mainly influenced by the concentration gradient across a functionally " homogeneous " bulk cuticle phase. This possibility is precluded by the following evidence:

(i) Concentrated aqueous solutions of protein and lipoid dispersant such as sodium cetyl sulphate are relatively non-toxic to blowfly larvæ, and fail to disrupt the epicuticle framework. After immersion in 25 % aqueous sodium cetyl sulphate for half an hour, blowfly larvæ are still active and resistant to pure methyl or ethyl alcohol.

(ii) Systems (*e.g.* oleic acid, oleyl alcohol, cetyl alcohol) which should induce emulsification of the lipoid and protein cuticle components exert a pronounced antagonistic action when incorporated in an external drug phase such as amyl alcohol, or ethyl alcohol-kerosene mixtures (*cf.* Table II).

The experimental evidence favours a two-dimensional surface diffusion along the Gibbs' layer or functional interfaces of the lipo-protein mosaic.

[11] Dennell, *Nature*, 1943, **152**, 50.

The physico-chemical factors which may influence this mode of drug access include :

(i) Concentration at the Gibbs' layer.
(ii) Spreading coefficient at the Gibbs' layer.
(iii) Physical nature of the adsorbed monomolecular film.
(iv) Functional viscosity of bulk lipoid phase.
(v) Functional viscosity of bulk protein phase.
(vi) Presence of other capillary active reactants at the Gibbs' layer.
(vii) Specific molecular interaction of drug molecules with functional cuticle components at the Gibbs' layer.

Under conditions in which the viscosity of the bulk lipoid phase is not a limiting factor in drug access, *i.e.* with pure drugs or drug-kerosene mixtures within the range $C_5$ to $C_8$, the biological activity of the alcohols present as an external drug phase falls rapidly as the series is ascended. This cannot be attributed to changes in *internal* molecular activity as measured by injection of the drugs into the hæmolymph. Since capillary activity increases in the direction $C_5$ to $C_8$, the relative degree of adsorption also increases in this direction. It is clear, from these considerations, that molecular drug concentration at the Gibbs' layer is not a limiting factor in drug access.

In homologous series, the spreading coefficient at the Gibbs' layer decreases as the hydrocarbon chains of the drug molecules become longer. This is due to an increase in the mutual cohesion between the molecules at the surface. Within the range $C_1$ to $C_8$ the adhesion between the hydrocarbon chains is relatively small, and the molecules from mobile " gaseous " films.[12] The adsorption of drugs at the functional liquid/liquid interfaces in the cuticle framework is primarily influenced by the work of adsorption at an oil/water interface. The rate of spreading or the mobility of an alcohol, *e.g.* octyl alcohol, at the oil/water interface may be slowed down considerably by increase in viscosity of (i) either of the bulk phases, or (ii) by the presence of an adsorbed interfacial film of moderately high capillary activity (*e.g.* cholesterol, cetyl alcohol, oleyl alcohol).

The above considerations suggest that the limiting factors which influence two-dimensional drug diffusion through the cuticle framework are :

(i) Polar interaction of the drug molecules with the protein components at the Gibbs' layer.

(ii) Non-polar van der Waals' interaction between the hydrocarbon chains of the drug molecules and the lipoid components at the Gibbs' layer.

(iii) Interfacial viscosity at the Gibbs' layer, which may be modified experimentally by the presence of substances of high capillary activity in the external drug phase. Selective adsorption of these substances results in a blocking of the interface, thereby producing a decrease in the accessibility to other components in the external drug mixture which are of lower surface activity.

Two-dimensional drug diffusion along the Gibbs' layer does not preclude three-dimensional diffusion through the bulk lipoid and protein phases, which probably is predominant with non-capillary active systems, but from this elucidation of the limiting factors in capillary active drug access, it is now possible to explain the gross divergence in drug access between the alcohols and fatty acids recorded in Fig. 1.

**Penetration of Alcohols.**—Owing to weak polar interaction of alcohols with the proteins of the cuticle, and weak non-polar interaction of the lower members ($C_1$ to $C_3$) with the lipoids, carrier activity of the drugs is relatively feeble within this range. As the series is ascended from $C_1$ to

[12] Adam, *The Physics and Chemistry of Surfaces*, London, 1941.

$C_5$, the increase in carrier activity owing to dispersant action of the alcohols on the lipoid phase results in an increase in functional cuticle permeability. A specificity factor appears to be involved in the non-polar interaction of the drug molecules with the lipoids of the cuticle, for dispersant action decreases as the series is ascended further from $C_5$ to $C_8$, within which range drug access also diminishes. It is possible, however, that with increase in chain length, the increase in the cohesion of the drug molecules becomes a limiting factor in mobility within the range $C_5$ to $C_8$.

**Penetration of Fatty Acids.**—The strong polar interaction of fatty acids with the protein components of the cuticle is sufficient to induce a selective penetration through the protein phase by the preliminary rupture or dispersion of salt interlinkage systems (*e.g.* $-\overset{-}{COO}\ \overset{+}{N}H_3-$) owing to competition of the polar groups of the drug molecules. Evidence for this assumption is provided by the penetration and expansion of synthetic monomolecular amine and protein films by fatty acids.[13] It is possible to visualise a competition between the lipoid and the protein components of the cuticle for the fatty acid drug molecules. Where chain length is short (*e.g.* formic acid) polar group interaction will be a limiting factor in drug access. Rapid penetration through the cuticle takes place through the protein phase since the weak short range van der Waals' attraction forces between the drug molecules and the lipoid phase are not limiting factors in drug mobility at the Gibbs' layer. As capillary activity increases, the drug molecules tend to become anchored at the external layers of the cuticle owing to a balance between the polar and non-polar interactions of the molecules with the lipo-protein associations at the Gibbs' layer. The stability of the lipo-protein-drug association reaches a maximum at $C_5$ (valeric acid) when drug access is at a minimum. From $C_5$ to $C_7$ this stability decreases as the influence of non-polar interaction begins to predominate over the competing influence of polar interaction. Dispersion of the lipoid phase now becomes a limiting factor in drug access, and increase in functional cuticle permeability accounts for the increase in drug reactivity within this range.

The dispersant action of drugs and fat solvent carriers on the cuticle is extremely complex. In lipo-protein associations, dispersant action must certainly involve both lipoid and protein components since these are invariably closely associated in living biological systems, and related changes in molecular orientation will take place depending on the elasticity of the bounding lattice framework. It is likely that the functional liquid/liquid interfaces in the cuticle framework are saturated with the relatively labile capillary active lipo-protein associations initially secreted by the internal epidermal cells. Primarily, dispersant action may involve a reversible reduction in the mutual cohesion of the labile lipo-protein associations at the Gibbs' layer. Monolayers of sterols are readily penetrated by short chain fatty acids or alcohols resulting in the formation of mobile "gaseous" films.[12] Prolonged association of the cuticle with drugs or fat solvents produces an irreversible increase in permeability, which may be correlated with displacement of the lipo-protein associations ; in this condition the cuticle is in a state of physiological unbalance, which is reflected by the increase in the rate of water loss. In the normal insect conservation of essential water is a major physiological role of the cuticle framework, and this is achieved by the modification of the extreme outer layer to form the epicuticle.

Induced access of drugs by non-polar solvents such as hexane, octane, or dodecane, may be ascribed largely to a decrease in the functional viscosity of the lipoid phase resulting in a large increase in functional cuticle permeability. The fact that induced penetration does not occur in an aqueous carrier medium suggests that the gel structure of the protein

[13] Cockbain and Schulman, *Trans. Faraday Soc.*, 1939, **25**, 716.

phase is relatively compact, and the possible swelling action of the carrier medium by participation in the protein phase, is insufficient to permit access of drugs even when the molecular volumes are small. Induced penetration occurs with polar carriers of moderate capillary activity (*e.g.* octyl alcohol) since the increase in drug mobility owing to dispersant carrier action is a more important factor in drug access than the opposing influence of carrier adsorption at the Gibbs' layer. Where the capillary activity of the carrier is high (*e.g.* cetyl alcohol, oleyl alcohol, cholesterol, or oleic acid), blockage of the Gibbs' layer by selective carrier adsorption accounts for the antagonistic influence of carrier activity on drug access.

## Drug Reactivity at the Site of Action.

If the valid assumption be made that the primary interaction of drugs injected into the hæmolymph takes place at specific lipoid or protein loci, and that the interactions at specific enzyme centres embedded in the lipo-protein associations occur secondarily, it becomes possible to correlate internal molecular drug reactivity with external drug reactivity, where diffusion to the site of action is a disturbing influence.

From Fig. 1 (*b*), it is seen that the relation between internal molecular activity and chain length of the homologous series of alcohols approximates to an exponential form which is characteristic of pharmacological data expressing equilibria between drugs and cells. This indicates that, where diffusion to the site of action is not a limiting factor in drug access, biological activity depends on the van der Waals' interaction of the non-polar portions of the drug molecules with the lipoid loci at the specific cell receptors.

The molecular isoactivity of the fatty acids as the series is ascended from $C_1$ to $C_5$ suggests that, within this range, biological activity is influenced chiefly by head group interaction of the polar portions of the drug molecules with the protein receptors at the site of action ; increase in length of hydrocarbon chain produces no increase in biological activity which is comparable with the logarithmic rate of change shown by the alcohols. Where chain length is short, the molecular activity of the fatty acids is much higher than that of the corresponding alcohol homologues. The influence of chain length is shown by the fact that at $C_5$ both series are isoactive, showing that a critical value in the van der Waals' interaction of the drug molecules with the lipoid centres has been attained. When the drugs are applied externally, this critical value is also reached at a chain length of $C_5$, but the selective molecular interaction of the drugs with the cuticle framework, owing to selective polar interaction of the fatty acids with the protein components, results in a *maximum divergence in relative drug reactivity* (Fig. 1 (*a*)) showing clearly the influence of a limiting diffusion factor.

## Molecular Interaction as a Factor in Narcosis.

The theory, first proposed by Overton and H. Meyer, that narcosis depends on the interaction of the drugs with the cell lipoids has recently been formulated in a more general manner by K. H. Meyer :[14]

" Narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipoids of the cell (or, to be more precise, in the lipoidic alcohols of the cell substance). This concentration depends on the nature of the animal or cell, but is independent of the narcotic."

Meyer points out that this rule has nothing to do with membranes, but is merely an expression of the fact that the irritability of the cell is diminished when the lipoidic alcohols of the cell become charged with a limiting threshold concentration of narcotic. The evidence provided was based on comparison of the isoactive concentrations of gaseous and water-soluble narcotics which produce narcosis when applied as an *external*

drug phase to mice and tadpoles respectively. From the experimental evidence obtained, Meyer concludes that there is no kind of correspondence between narcotic activity and surface activity, and that any theory which is based on a connection between these two is not in agreement with the facts.

On the other hand, the regularity was observed that the concentration in oleyl alcohol as set up in equilibrium with the effective concentration in the medium (air or water) is always constant, or nearly so. Correlation of biological activity with solution in the cell lipoidic alcohols was based in this analogy of drug phase distribution relationships.

Clark and other workers have stressed the fact that the regularity with which the pharmacological activity of narcotics increases with length of hydrocarbon chain may be explained most readily by the hypothesis that the narcotics are adsorbed on surfaces in the cells, although most of the results are consistent with the alternative hypothesis that drugs dissolve in and alter the cell lipoids.[1e, f, i, 8]

When the experimental facts of observation are in accord with either of two fundamental physico-chemical laws, *i.e.* (1) distribution between immiscible phases depending on differential solubility, or (2) distribution depending on adsorption at interfaces, the apparent ambiguity disappears in a wider statement of theory, or in more specific definition of the variable factors in the particular systems. The chief difficulty in the interpretation of the significance of pharmacological data has been the lack of precision in the experimental evidence as to the nature of drug-biological system phase distribution relationships. This ambiguity disappears when the nature and site of drug interaction is more clearly defined. Comparison of the biological responses produced by injections of aqueous solutions of alcohols and fatty acids into the hæmolymph of *Phormia* larvæ shows :

(i) The narcotic symptoms produced by injection of the lower members $C_1$ to $C_3$ in both series of drugs are relatively ill-defined. Drug tolerance is small within this range. Drug concentrations above the threshold narcotic values produce rapid pathological changes in the tissues resulting in death shortly after drug injection.

(ii) *Intensity* of narcosis and tolerance to higher concentrations than the threshold narcotic dosages reaches a maximum at $C_5$.

(iii) With further increase in chain length ($C_6$ to $C_8$) the intensity of narcosis decreases in both series. With the alcohols, this becomes most apparent at $C_6$ ; *saturated* aqueous solutions of higher homologues are relatively inactive when injected, and fail to produce complete paralysis. With the fatty acids, biological activity falls off at a slightly earlier stage ($C_5$) ; saturated solutions of higher members are relatively inactive.

If biological activity is measured by the *intensity* and *duration* of the symptoms of paralysis produced by drug injection, correlation between the molecular activities of the alcohols and fatty acids is shown at a chain length of $C_5$. The relative differences in activity between the lower members in both series may be attributed to chemical reactivity due to head group interaction of the drug molecules with the protein receptor groups in the biological system. At $C_5$, van der Waals' interaction between the hydrocarbon chains of the drug molecules and the lipoid receptors becomes a limiting factor in drug reactivity *in the particular systems used*. This suggests that narcosis is influenced by specific non-polar stereochemical relationships between the drug molecules and the lipoid receptors at the site of action. The marked analogy between the *nature* of the interactions of drug molecules with the lipo-protein associations of the cuticle framework and the internal receptors at the site of action suggests that adsorption at functional lipoid/protein interfaces within the insect is a major factor in biological activity.

The dominant influence of a diffusion factor in measurements of drug reactivity is shown clearly by the following experimental evidence:

During the mature larval stage, the cuticle of the blowfly *Phormia terrænovæ* increases greatly in thickness, and the relative resistance to drugs such as alcohols and fatty acids applied externally increases during this stage. The relative resistance to drugs applied internally by injection into the hæmolymph shows no marked change as pupation approaches. Similar experiments on the related series of blowfly larvæ

(*a*) *Calliphora erythrocephala* (*b*) *Phormia terrænovæ* (*c*) *Sarcophaga falculata*

show that functional cuticle permeability and relative susceptibility to drugs applied externally decreases in the direction $(a) \rightarrow (b) \rightarrow (c)$. When the drugs are applied internally by injection, molecular isoactivity is shown by corresponding homologues in all three species of test insect. These experiments show that *the "biological activity" of a drug has little quantitative significance unless the mode of drug application and the drug-biological system phase distribution relationships are clearly defined.*

When *Calliphora erythrocephala* larvæ are immersed in pure alcohols, penetration through the cuticle takes place more rapidly than through the cuticle of *Phormia* larvæ (Fig. 1 (*a*)). Since the internal molecular activities are of a similar order, it is permissible to compare the relative external activity per molecule by the reciprocals of the survival times when the insects are immersed in pure drugs, taking as unity the survival time in pure methyl alcohol for each organism. The results are recorded in Table III. The results show that with both test insects molecular activity increases from $C_1$ to $C_5$ as the series of drugs is ascended, but the *rate of increase in biological activity is much more pronounced* with *Calliphora* than with *Phormia*. From $C_5$ to $C_8$ biological activity falls in both systems.

TABLE III.—RELATIVE EXTERNAL BIOLOGICAL ACTIVITIES OF HOMOLOGOUS SERIES OF ALCOHOLS TO INSECTS OF DIFFERENT SUSCEPTIBILITY.

| Length of Chain. | Survival Time (mins.). | | Molecular Activity. | |
|---|---|---|---|---|
| | *Phormia.* | *Calliphora.* | *Phormia.* | *Calliphora.* |
| $C_1$ | 190 | 125 | 1·00 | 1·00 |
| $C_2$ | 160 | 93 | 1·18 | 1·34 |
| $C_3$ | 135 | 25 | 1·41 | 3·58 |
| $C_4$ | 85 | 7 | 2·23 | 17·9 |
| $C_5$ | 50 | 5·5 | 3·8 | 25·0 |
| $C_6$ | 125 | 6 | 1·52 | 20·8 |
| $C_7$ | 150 | 7·5 | 1·26 | 16·7 |
| $C_8$ | 170 | 9·5 | 1·12 | 13·2 |

These results have an important bearing on interpretations of drug specificity in pharmacological systems. It has generally been assumed that drug specificity depends mainly on the nature of the molecular interaction of the drug with the ultimate cell receptors in the biological system. *Where differences in drug access are limiting factors in biological activity in a range of biological systems, the relative biological activities of a range of drugs measured with one particular biological test system may differ considerably from the corresponding biological activities measured with another system, even though the molecular activities at the sites of action in the systems are similar.* This may be attributed to the selective action on drug access of drug molecular interaction with intermediary biophases in the biological system which link the drug "source" with the drug "sink."

Still further complexities appear when the range of biological systems is extended to include other insects. By far the majority of insects (*e.g.* adult Diptera, larval and adult stages of Coleoptera, Lepidoptera, nymphal and adult stages of Orthoptera, Hemiptera) become rapidly immobilised when immersed in pure drugs such as alcohols and fatty acids, and bio-assay is impracticable in such systems owing to the rapidity of response produced. Even fat solvents such as kerosene, which are relatively non-toxic to dipterous blowfly larvæ, are rapidly toxic when presented to other insects as a relatively large external drug phase. These differences cannot be ascribed entirely to diffusion factors. Kerosene is non-toxic to blowfly larvæ when injected into the hæmolymph at dosages as high as 30 mg./100 mg. body weight. In fact, in the homologous series of paraffins, there is a sharp cut-off in internal and external toxicity as the series is ascended within the range $C_9$ to $C_{11}$. Below this range, rapid paralysis is induced within 1 to 2 minutes either by external or by internal drug application. Above this range, the insects survive from 1 to 3 hours. With other insects the biological " cut-off " in toxicity takes place higher in the series. Even high boiling fractions of the medicinal paraffin type produce rapid paralysis when applied externally or internally, although in systems of this type, complications arise from the rapid increase in viscosity as the series is ascended. Where the test insect is susceptible to carrier toxicity, bio-assay of drug activity becomes more difficult, and involves a reduction in the relative volume of drug phase applied to the insect externally. For example, the drug mixture may be first deposited on a porous substrate, where a proportion of the mixture becomes immobilised by absorption or adsorption processes. The deposit of drug mixture may be adjusted to form a thin film in the surface of the framework. " Film activity " may be measured by introducing suitable test insects on to the substrates which are treated with a range of deposits and drug concentrations.[14] In systems of this kind, transport of the drug to the test insects is mainly influenced by the capacity of the localised drug film/test insect interface for drug transmission.

## Carrier Activity of Aqueous Drug Solvents.

It has been pointed out that water has a negligible carrier activity in the presence of drugs of low molecular activity, such as ethyl alcohol. With drugs such as pyrethrins, molecular activity is extremely high, and these toxic principles form the chief basis of existing insecticidal mixtures. No synthetic insecticide has yet been prepared which approaches pyrethins in biological activity. Pyrethins are powerful nerve poisons, which interact specifically with the peripheral and central nervous systems of insects. The molecular structures of the pyrethrin I and II molecules are exceedingly complex, and owing to the instability of the drugs, uncertainty still exists as to the precise structure, although valuable contributions to this aspect have been made recently by Gillam and West.[15]

Drug reactivity depends on multipoint head group and non-polar interaction with specific receptors in the nervous system. This interaction is primarily reversible, but eventually leads to dispersion or disruption of the nerve cells, which may be shown by appropriate histological techniques. Where the carrier system consists of two miscible phases (i) an organic solvent (ethyl alcohol), and (ii) water, the bulk homogeneous carrier phase is relatively non-toxic to blowfly larvæ, such as *Calliphora*, for all proportions of the components. If pyrethrins are present in this mixed carrier system, *the threshold concentration of pyrethrins which induces primary symptoms of paralysis in the insect decreases as the proportion of water in the system increases.*

[14] This system has been used as a basis for a standard method of insecticidal bio-assay (Hurst, *Nature*, 1943, **152**, 400).

[15] Gillam and West, *J. Chem. Soc.*, 1942, **139**, 487, 673.

The interpretation of this " aberrant " example of drug concentration-biological activity relationship is possible from some experiments which have been carried out on the Adam-Langmuir trough.[12] The capillary-active pyrethrins are only very slightly soluble in water. The drug molecules form a highly mobile " gaseous " or " liquid-expanded " monolayer at the air/water interface. This is due to the multipoint polar attraction of the orientated molecules for the water phase, and also to the small mutual cohesion of the hydrophobic portions of the molecules. The non-toxicity of a given concentration of pyrethrins in a pure ethyl alcohol carrier is due to the fact that adsorption from the organic solvent does not take place. Traube's rule does not hold for adsorption from organic solvents. The short range forces between hydrocarbon portions of molecules are much feebler than the fields of force associated with polar portions of the molecules ; hence positive adsorption of a capillary active drug molecule at an organic solvent/air interface or organic liquid/liquid interface does not occur, since this would involve an increase in free energy of the system.

Since lipoids are present at the surface of the cuticle of blowfly larvæ, the contact of an external water phase with the cuticle will immediately introduce a system of *functional* lipoid/water interfaces at discrete loci on the cuticle surface. When water is added to an external drug phase consisting of a solution of pyrethins (0·2 %) in ethyl alcohol, adsorption of pyrethins takes place at the functional lipoid/water interfaces introduced into the system by the addition of water. The increase in concentration of pyrethrins presented to the insect at this two-dimensional interface is greater than the corresponding decrease in concentration in the bulk external drug mixture owing to the diluent action of water. Owing to the high molecular reactivity of the drug molecules, cuticle permeability is no longer a limiting factor in drug access at certain initial concentrations in the original ethyl alcohol carrier phase.

## Biological Activity of Drugs Applied as External Two-dimensional System.

When pyrethrins are applied in an organic solvent such as kerosene, penetration of the drug through the cuticle takes place rapidly, owing to high carrier activity. The factors which influence this " induced penetration " have already been considered in other drug systems. With the following range of test insects, relative resistance (measured by application of insecticides as three-dimensional " films ") increases in the order

| | | | | |
|---|---|---|---|---|
| (*a*) *Phormia terrænovæ* (adult) | → | (*b*) *Calliphora erythrocephala* (adult) | → | (*c*) *Sarcophaga falculata* (adult) |
| (*d*) *Tenebrio molitor* (adult) | → | (*e*) *Tenebrio molitor* (larva) | → | (*f*) *Calandra granaria* (adult). |

When pyrethrins are adsorbed as a unimolecular film at the air/water interface, the relative activities of the drug molecules at the Gibbs' layer may be measured by using test insects as biological indicators. The insects are suspended at the Gibbs' layer, or immersed in the underlying aqueous bulk phase. By carrying out these experiments in the Adam-Langmuir trough, the concentration or activity of the adsorbed monolayer of pyrethrins may be varied experimentally by means of moveable waxed barriers. The results of such experiments have shown that (i) the biological activity of pyrethrins in the bulk aqueous phase is negligible, and (ii) *the relative order of resistance of insects to pyrethrins applied as a two-dimensional film corresponds to the order of resistance when the drugs are applied as a three-dimensional phase in an organic solvent.*

**Pharmacological Action in relation to Fundamental Biological Pattern.**

From these results the following conclusions, which are of general biological significance, may be drawn :

(1) Measurement of the biological activity of capillary active drugs by determination of the active concentrations in an aqueous carrier medium is not necessarily an indication of the active concentrations at the carrier/biological system interface owing to the possibility of selective adsorption at the functional lipoid/water interfaces induced by contact of the carrier with the biological system.

(2) Wherever an aqueous phase participates functionally, or is introduced into a biological system, adsorption of capillary active drugs at biophases in contact with this aqueous phase may be a limiting factor in drug reactivity.

The general implications of these principles are immediately obvious, for lipoid/protein interfaces, which are fundamentally of the oil/water type, are of universal occurrence in biological systems. The carrier activity of an external aqueous phase for pyrethrins is parallelled by the carrier activity of the internal body fluids of the insect when capillary active drugs are injected into this biophase. This accounts for the typical Traube series formed by the molecular activities of the homologous series of monohydric alcohols when injected into the hæmolymph of blowfly larvæ (Fig. 1 (*b*)). Moreover, the generalisation known as Richardson's rule, which states that for any one organism, biological activity in a homologous series of drugs increases with increase in molecular weight may be regarded simply as an expression of the escaping tendency of each successive homologue from an aqueous phase to the lipoid receptors in the system. More specifically, the logarithmic relation between biological activity and length of carbon chain suggests that, in systems in which this relation holds, selective molecular interaction with an intermediary biophase is not a limiting factor in drug access, and also that biological activity is influenced by a relatively specific van der Waals' interaction of the drug molecules with the lipoids at the site of action. This relation holds for a wide range of straight chain compounds. The relatively high molecular activities of the lower fatty acids $C_1$ to $C_5$ when injected into the hæmolymph of blowfly illustrate the " swamping " effect of polar interaction on non-polar interaction, and it is likely that the apparent lack of correlation between the biological activities of the lower members in homologous series of drugs and chain length is due to a similar effect, which may be accentuated when drug access to the site of action is influenced by selective molecular interaction with a bounding membrane system. These considerations help to explain the chief difficulty which has hitherto precluded a more general acceptance of the adsorption theory, namely that the parallelism between pharmacological action and capillary activity does not hold when different series are compared.

The wider implications of these principles become apparent from examination of the large body of pharmacological data which shows that, in many systems, the specific and non-specific reactivities of aqueous drug mixtures depend on drug interaction at biophases in which diffusion processes are not limiting factors in drug access. For example, immersion and micro-injection experiments on unicellular organisms have shown that narcotics and more chemically active drugs (*e.g.* cyanides) act specifically on the surface of protozoa such as amœbæ or paramœcia.[16] When a multicellular tissue such as frog's heart is exposed to an aqueous solution of methylene blue, pharmacological action involves a surface atropine-like action which occurs before penetration of the dye into the heart cells takes place. Similarly, the antagonistic action of methylene blue on the

[16] (*a*) Hiller, *Proc. Soc. Exp. Biol. Med.*, 1927, **24**, 427, 938. (*b*) Brinley, *J. Gen. Physiol.*, 1928, **12**, 201 ; (*c*) *Proc. Soc. Exp. Biol. Med.*, 1928, **25**, 305.

specific inhibitory activity of acetyl choline occurs before the heart cells are stained by the dye.[17]

In systems of this kind, drug reactivity will be influenced primarily by distribution between an aqueous phase and a heterogeneous surface which is the site of action. Since it is reasonable to assume that at least some of the lipophilic elements at the site of drug action will be directly in contact with the bulk aqueous phase, the presence of this phase introduces functional lipoid/water interfaces into the system. Where the drugs are non-capillary active, interaction with the lipoid centres will depend on differential solubility between the bulk aqueous and lipoid phases. For capillary active drugs, molecular interaction will be primarily related to the work of adsorption at an oil/water interface. If the lipoid loci at the site of action are of unimolecular thickness, the distinction between differential solubility and adsorption factors tends to disappear, since the distribution equilibria in both systems will depend on drug fixation at a two-dimensional interphase.

The analogy between pharmacological action in living systems and drug phase distribution equilibria in heterogeneous oil/water synthetic systems only holds for certain pharmacological systems in which an aqueous phase is an essential component, and a diffusion process is not a disturbing influence in drug access. These simple model systems cannot be used to explain the gross divergence in drug access between corresponding homologues of alcohols and fatty acids (Fig. 1). A *specificity* factor is clearly involved, and the key to the fundamental nature of this factor is obtained from a comparison of a wide range of pharmacological systems. All biological systems contain protein and lipoid elements, which are functionally *fixed* structures in which physiological balance may be regulated by the functional participation of secondary biophases (*e.g.* enzymes) in the systems. Structurally, lipo-protein systems are more analogous to visco-elastic gels than to heterogeneous liquid systems.

The conception of structure in a biophase leads to the possibility of selective drug access owing to specific molecular interaction of the drug molecules with the *fixed* active or receptor groups in the bulk biophase. The drug or carrier may alter selectivity by inducing reversible or irreversible changes in the lipo-protein associations, or more specific association of the drug with the lipoid and protein components may take place so that biological activity is obscured by selective drug access. In liquid systems, selectivity is a function of drug mobility in a bulk liquid phase. Although liquid/liquid systems are of common occurrence in biological systems, the morphological disposition of these liquid biophases is primarily influenced by association with relatively fixed structural biophases. In these heterogeneous liquid-solid systems, it is difficult to assess the relative influence on drug access of the discrete component phases which are functionally inseparable. In so far as gross physical properties (*e.g.* viscosity) may be limiting factors in drug access, the conception of "functional viscosity" gives a measure of the average yield value of the biophases to drug diffusion pressure.

The establishment of the fundamental relations between biological system, drug, and carrier has depended on the choice of suitable systems. This selection was made *after* preliminary examination of other related and apparently unrelated systems. The effects of "induced drug penetration" and selective drug access are shown by a wide range of insects. With aquatic *Chironomus* larvæ, penetration of ethyl alcohol is induced so rapidly by a carrier such as kerosene, that the insect swells and bursts within a few *seconds* after immersion in the drug mixture.

During the hardening of insect cuticle, there is a decrease in absolute and selective permeability; the relative degree of drug penetration induced by fat solvents is less than in insects with soft cuticles. This

[17] Cook, *J. Physiol.*, 1926-7, **62**, 160.

may be correlated with the "tanning" of the chitin-protein complex by phenolic and quinonoid substances which link or bond specific acceptor groups,[8c, 9a, b, 18] and which results in the formation of a relatively rigid chemically-linked three-dimensional cuticle lattice framework.

As the range of biological systems is extended, it is seen that the permeability of the insect cuticle has many features characteristic of exoskeletal structures in Arthropods in general. Drug access through the cuticle of microscopic organisms such as mites is increased by non-polar solvents such as kerosene. It is interesting to notice that, even with these minute organisms, carrier toxicity of paraffins such as dodecane is relatively small. These test systems are particularly suitable for the investigation of drug reactivity, since the relatively "untanned" integument interacts readily with drugs such as amines or phenols, thereby providing a sensitive indication of the contributory influence on gross biological activity of polar interaction. Owing to the small size of these organisms, the effects of drug depletion in the external drug phase are relatively negligible. By way of contrast, the somewhat specific features of *selective directional* permeability which have been described by Yonge[19] for the *internal* integument which lines the foregut of the lobster *Homarus* are, in fact, characteristic of Arthropod external integument. In this connection, there is a marked physiological analogy between the lipophil "cuticle" which lines the internal integument and the outer epicuticle of insects. Both these bounding membranes appear to be morphologically adapted to serve as regulatory selective diffusion systems.

Similar properties are shown by the integument in other groups of Invertebrates. For example, the integument of the nematode worm *Ascaris*, which lives as a parasite in the aqueous lumen of the alimentary canal of Vertebrates such as the pig, is fundamentally of the same morphological and physiological *pattern* as insect cuticle. The thin outer layer of Ascaris cuticle, which may be detached mechanically from the underlying layers, is analogous to insect cuticle. Penetration of drugs such as alcohols may be induced by carriers such as kerosene or hexane. The worms swell and burst when placed in a mixture of hexane and methyl alcohol. As the alcohol series is ascended, there is a sharp "cut-off" in selective drug access. The worms *shrink* owing to dehydration when immersed in mixtures of ethyl alcohol and kerosene or hexane, and a similar effect is shown with drug mixtures containing higher homologues. Shrinkage also occurs in pure ethyl alcohol. A comparison of the carrier activities of aqueous and fat solvent media reveals some striking results, which are not obvious with the more highly "tanned" insect cuticle. In the presence of water, the rate of dehydration of the worm decreases with increase of the proportion of water in the external ethyl alcohol-water mixture. In the presence of kerosene, the rate of "exosmosis" *increases* with increase in the proportion of oil in the external medium. These results can clearly not be correlated with simple distribution between the internal body fluids and the external drug phase, for in the ternary homogeneous mixture ethyl alcohol-kerosene-water, the solubility of water decreases with increase in the relative proportion of the oil component. An explanation is provided by analogy with the action of oils on the permeability of the insect cuticle to water. In these systems the participation of oils such as kerosene in the lipoids of the cuticle induces a decrease in functional viscosity of this phase which results in an increase in the mobility of water molecules in the outer bulk framework of the epicuticle. Molecular interaction of the kerosene carrier with the lipoid centres in the *Ascaris* integument results in a similar "opening-up" of membrane lattice structure. The rate and extent of exosmosis depends on the water storage capacity of the external drug phase.

[18] Pryor, *Proc. Roy. Soc., B*, 1940, **128**, 378.
[19] Yonge, *ibid.*, 1936, **120**, 15.

The sensitivity of the *Ascaris* cuticle to changes in the external drug phase is an excellent guide to the interpretation of drug phase distribution relationships in insects. The hydrophilic protein components in *Ascaris* cuticle comprise a greater proportion of the bulk framework than in insect cuticle, and in the homologous series of alcohols, drug mobility at the functional lipoid/protein interfaces becomes a limiting factor in drug access as the alcohol series is ascended from $C_1$ to $C_2$. This "cut-off" may be demonstrated in other systems. The cuticle of the blowfly larva *Calliphora* represents a transitional stage between the relatively "untanned" *Ascaris* cuticle and the "hard" cuticle typical of adult Coleoptera. In the soft insect cuticle (*e.g. Calliphora*), the "cut-off" in the relative degree of drug access which is induced by fat solvents occurs as the alcohol series is ascended from $C_3$ to $C_4$. The hardening of insect cuticle is associated with a decrease in hygroscopicity of the protein phase, involving a reduction in functional free volume of this phase. Structurally, the hard cuticle consists of a relatively rigid open chemically-linked lattice framework which encloses the labile "bonding" phase, of which the hydrophobic lipoid components play a predominant role in cuticle permeability. As the cuticle hardens after a moult, the position of the "cut-off" in the relative degree of induced drug penetration proceeds *down* the homologous series of alcohols until it reaches the range $C_1$ to $C_2$. This indicates that with decrease in the van der Waals' forces of adhesion of the drug molecules to the lipoid centres in the cuticle drug mobility at the Gibbs' layer is a limiting factor in drug access which overshadows drug solubility in the bulk lipoid phase. The rapid access of the corresponding fatty acid homologue, formic acid has been discussed previously; here drug access is facilitated further by selective polar interaction with the protein phase.

When other drug systems are compared, the distinction between the soft *Ascaris* cuticle, and the hard insect cuticle, becomes more apparent. *Ascaris* cuticle is readily permeable to water—a necessary biological adaptation to the aqueous environment in which the parasite lives. The protein components interact strongly with amines and phenols, the activity of these drugs being much enhanced by the presence of kerosene, suggesting a lipo-protein mosaic structure in the cuticle. When the isolated cuticle is "tanned" by prolonged immersion in formalin or tannic acid solution, this selective permeability diminishes. This offers a marked analogy to the changes which take place in living insects during the hardening of the cuticle after a moult.

Similar factors influence drug access through pulmonary and muscle epithelia, nerve tissue linings, and similar structures in Vertebrates such as the pig or sheep. The membranes may be examined experimentally when attached to tubes to form simple osmometers.

The red cell or erythrocyte has been extensively used as an experimental system in quantitative pharmacological studies.[20] While the precise molecular ultrastructure of the red cell is still not clearly defined, there is evidence that the lipoid may be bound to the protein so that there is an orientation of lipoid at particular loci around the protein molecules rather than a formation of a continuous homogeneous surface layer.[21] This suggests that the red cell envelope consists of a lipo-protein mosaic. The primary association of fat solvents with these lipo-protein centres produces reversible changes in molecular orientation of the cell surface envelope. This is shown by the reversible nature of the disc-sphere transformations which may be produced by the primary interaction of

[20] Ponder, *The Mammalian Red Cell and the Properties of Hæmolytic Systems*, Berlin, 1934.

[21] (*a*) Parpart and Dziemian, *Cold Spr. Harb. Symp., Long Island Biol. Ass.*, 1940, **8**, 17. (*b*) Ballantine and Parpart, *J. Cell. Comp. Physiol.*, 1940, **16**, 49. (*c*) Ponder, *J. Exp. Biol.*, 1942, **18**, 257; (*d*) *ibid.*, 1942, **19**, 215, 220.

fat solvents with the red cell. Prolonged interaction of fat solvents disrupts or disperses the lipo-protein associations, and results in eventual hæmolysis.

Further extension of the range of biological systems shows that the analogy between insect cuticle and other lipo-protein associations bridges the gap between the animal and plant systems. This parallelism is revealed from a comparison of the outer "cutinised" layer of plant epidermal tissues and the external epicuticle of insects. For example, the penetration of ethyl alcohol through the outer skin of the grape or tomato may be induced by fat solvents such as kerosene.[22] Prolonged association with fat solvents produces a dispersant action which is similar to that which takes place in insect cuticle and in other animal membranes.

The range of drugs which show the effects of induced penetration in the presence of fat solvents of high carrier activity include feebly dissociating capillary active alcohols, ketones, amines, and phenols. The drugs penetrate as unionised molecules. This is in agreement with their greater activity in solvents of low dielectric constant, and is also consistent with maximum adsorption at the Gibbs' layer.

Drug access is most facilitated by non-polar fat solvents such as aliphatic straight chain paraffins, cyclic hydrocarbons such as cyclohexane, methylcyclohexane and dimethylcyclohexane, and relatively simple solvents such as carbon disulphide. These effects of carrier activity are quite distinct from carrier toxicity. which may, however, contribute to gross toxicity. Induced drug penetration is shown to a lesser degree by aromatic compounds such as benzene, toluene, xylene, pseudocumene, and mesitylene. The relative degree of induced drug penetration is still less pronounced with unsaturated aromatic carrier media such as indene or coumarone. These effects may be attributed to the fact that the aromatic solvent molecules are more polarisable than those of the aliphatic hydrocarbons, and this tendency towards capillary activity is still further accentuated with increase in the degree of unsaturation of the solvent molecules. In some systems, capillary active solvents such as oleic acid or sesame oil may reduce the carrier activity of a kerosene medium for highly reactive drugs such as pyrethrins owing to selective adsorption and blocking of the Gibbs' layer. But where an "overlap" between primary responses, such as paralysis, and secondary irreversible symptoms of joint toxic action occur, capillary active solvents may be more efficient "activators" for pyrethins than non-polar solvents, which mainly influence functional cuticle permeability. Measurement of "activator activity" depends on a precise definition of the stage at which gross biological responses are measured.

This discussion of the fundamental analogies between different pharmacological systems has shown that correlation of biological activity with simple drug distribution between heterogeneous phases may be greatly extended if more specific factors which influence molecular interaction in monolayers are taken into consideration. In this way the more general concepts of Overton-Meyer and Traube are no longer conflicting, but may rather be regarded as expressions of molecular interaction with the biological system which become mutually related when the precise nature of the drug-biological system phase distribution relationships are defined experimentally in an appropriate range of pharmacological systems.

In conclusion, it may be pointed out that the principles which have been elaborated in the present work apply not only to drug systems applied as liquids, but also to drugs applied in the vapour phase as fumigants. It is interesting to record that the main lines of enquiry along which the present investigation has proceeded were initiated by the discovery that the non-toxic non-polar components in various samples of heavy naphtha, which is a complex mixture of coal-tar derivatives, did not "obey the

[22] Hurst, *Nature*, 1941, **147**, 388.

rules " by acting as diluents for the more toxic capillary active components present, such as indene, coumarone, and cresylic acids, but actually enhanced the biological activity of these components.[6] This may be attributed to the condensation and functional participation of the drugs in the cuticle framework. A transitional system between liquid and gaseous drug systems may be illustrated by the " sensitization " of insects to fumigants which takes place when the insects are subjected to a preliminary treatment of liquid non-polar solvent such as kerosene. Owing to an increase in the functional permeability of the cuticle, the treated insects respond to a lower threshold concentration of fumigant than the untreated insects.

During the course of this work, many analogies between drug access in the living insect and drug access through artificial membranes such as rubber, collodion, or gelatine have been found.[22] But these analogies are only valid for a restricted range of drugs and biological systems, and become of significance when correlated with a range of pharmacological systems in which physico-chemical factors which influence drug phase distribution relationships are considered in relation to the specific physiological factors which primarily govern the nature of the biological responses produced.

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## *GENERAL DISCUSSION*

**Prof. Rideal** (*Cambridge*) asked whether the morphological arrangement of the lipo-protein mosaic in insect cuticle could favour lateral drug transmission through the cuticle framework.

**Dr. H. Hurst** (*Cambridge*), in reply, said : It is likely that the mosaic structure of the cuticle provides a network of two-dimensioned pathways which facilitate drug transmission both across and along the membrane. This may be demonstrated experimentally with pyrethrins, which are specific insecticidal nerve poisons. When pyrethrins are applied internally by injection, the drug is rapidly transported to the nerve tissues by the circulatory action of the hæmolymph, and complete paralysis follows almost immediately. This is manifested by a *simultaneous* loss in muscular coordination of the limbs of the insect. External local application of the drug results in the initiation of a well-defined chain of responses : (i) local paralysis at site of application ; (ii) progressive extension of zone of paralysis ; (iii) complete paralysis, similar to the condition induced by internal drug administration.

The cuticle framework is associated with a peripheral nerve network, which communicates internally with the central nervous system. The appearance of local symptoms of paralysis following local external drug application, followed by the progressive extension of the zone of paralysis suggests that primary drug fixation involves the association of the drug with the peripheral nerve network followed by the ultimate transmission of the drug along the nerve connectives to the central nervous tissue. The rapid paralysis which follows internal drug administration can be attributed to the carrier activity of the hæmolymph, in which convection ensures uniform drug distribution to the nervous tissues. Since the bulk cuticle framework is essentially a convection-proof barrier, the relatively rapid extension of the zone of paralysis following local external drug

application cannot be ascribed solely to local drug penetration through the cuticle into the hæmolymph followed by convection transport in this substrate, a process which would favour rapid simultaneous paralysis after the initial penetration of the drug through the cuticle had taken place. Neither can the progressive nature of the biological responses produced be explained by lateral drug diffusion through a cuticle framework in which discrete lipoid channels run transversely across the cuticle, since the two-dimensional lipo-protein discontinuities in this direction would involve a relatively slow three-dimensional diffusion across intermediary lipoid or protein phases.

This evidence indicates a continuity of lipo-protein interfaces along the membrane framework. The relatively abrupt morphological transition from a lipoid-rich epicuticle outer layer to the underlying hydrophilic chitin-protein layer would also favour selective adsorption and two-dimensional drug diffusion along the outer layer of the cuticle framework.

It is interesting to note the analogy between the insect cuticle and the bounding membranes of nerve fibres, where the presence of bounding layers of radially orientated lipoid molecules provides a pattern of interfaces which would favour rapid drug transmission along the outer nerve sheath.[23] In addition, the effects of induced drug access which may be obtained with isolated nerve sheath preparations suggest that narcotic action may well be associated with the reversible functional changes in free volume of the lipoid centres ; in this way corresponding changes in the capacity for ionic transmission of the nerve sheath, or in more specific molecular orientation of active patches would influence normal irritability. This simple conception conforms well with a large body of experimental data which shows that similar narcotic action may be produced by fat solvents of diverse chemical composition.

[23] Schmitt and Bear, *Biol. Rev.*, 1939, **14**, 27. Schmitt and Palmer, *Cold Spr. Harb. Symp., Long Island Biol. Ass.*, 1940, **8**, 94.

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# SOME PHYSICAL CHEMICAL PROPERTIES OF BIOLOGICALLY ACTIVE MOLECULES.

BY J. H. SCHULMAN.

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(*a*) *The physical chemical properties* that will be chiefly dealt with in this paper are those governed by short range forces, such as polar association or induced polar association as designated by Van der Waals forces.

These are the forces that become primarily involved when one molecule approaches another situated in a structure as in a membrane or site of action as in a nerve sheath, or by direct association in a plasma, or furthermore are directly responsible for the structure and viscosities of the membranes or plasma themselves. These forces are governed very simply by Coulombs' law and for the non-polar or induced polar forces by London's theories. Overton and Meyer on oil solubility and Traube on surface activity of molecules in relation to their biological activity are specialised aspects of this theory.

(*b*) *Biological activity* as described here is related to the concentration in which a certain chemical produces a standard biological response, there is thus a time factor which also has to be standardised. Enhancement of the activity by means of mixtures or adjuvants will not be discussed here. The chemicals which will be chiefly discussed are those that are lipoidal (hydrocarbon) in nature or possess a large hydrocarbon group, or proteins or mixtures of both, *i.e.* lipo-proteins. Although the physical chemical

properties which will be discussed in detail refer equally well to a lipoidal as to a protein molecule, the chemicals discussed will be divided into the above categories.

There are a number of complicating factors in assessing biological activity of a molecule which must be carefully sorted out before applying any rules to any specific system. For example, a molecule may have to penetrate a membrane, or associate with another molecule (carrier molecule) before reaching the site of action, or the molecule having become dispersed in the reacting system by the above means then has to be reacted upon by ferments, and it is the break-down products which produce the reaction. So a molecule may have to contend with membrane permeability, dispersion in the system by complex formation with a carrier molecule, or adsorption on to a colloid carrier (*i.e.* emulsion interface) and then attack by a ferment to form the actual reacting chemical *in situ* where it is required. A great number of molecules the chemistry of which will be discussed, answer in one way or another to the above criteria.

Dr. Hurst gives a good example of the effect of penetration of a membrane using certain insects as a test mechanism. The insect may either be immersed in the chemical or the chemical be injected into the insect, thus eliminating the effect of the membrane, or it is possible to treat certain chemicals like the cancer-giving hydrocarbons which would appear from the physical chemical behaviour to be inert biologically, with ferments or U.V. radiations *in vitro* and obtain the biological action from the break-down products. Thus it is possible to separate to a certain extent the various factors other than dispersion or carrier, which go to make up the standard biological response.

## Physical Chemical Characteristics.

The chemical groups involved in association between molecules are the hydrocarbon groups, polar groups and the ionic groups. Automatically the spacing and stereochemical interrelations between these groups must and can be shown to play a very important part in the resultant force of association between the molecules. The associating forces are directly proportional to $1/r^7$, $1/r^4$ and $1/r^2$ ($r$ = distance between groups) respectively (and $1/r^3$ for an ion dipole association). This shows how sensitive the association forces are to mutual orientation and adlineation between the molecules, thus any slight change in ionisation, dipole charge, number of carbon atoms, distance and spacing between the associating groups can make or break a complex, thus giving them their high degree of specificity. The monolayer technique (surface potentials and surface pressures) is a very convenient tool to measure the associating forces between all these groups in great detail, and it can be quickly shown that the interactions measured at the interface also take place in three dimensions. An interface is convenient for measuring these forces owing to the strong asymmetrical field which separates the polar from the non-polar groups, thus allowing them to interassociate, either inter- or intra-molecularly.

It can be shown that an alipathic hydrocarbon chain of $18(CH_2)$ is approximately equivalent in force of association with another hydrocarbon chain, both in the trans configuration, as an ion dipole association, both being of the order of 15,000 cal. The hydrocarbon association is markedly weakened when $CH_2$ groups are arranged in the cis form, thus preventing adlineation between the hydrocarbon chains. Likewise the polar association is broken down when polar groups are changed, for example from a $SO_4^-$ ion with an —OH dipole to an —$OOCH_3$ polar group, or $NH_3^+$ with an OH polar group to an $N(CH_2)_3^+$ with an OH polar group. The methyl groups in the latter case keeping the $^+$ion from approaching the OH polar group as close as in the unsubstituted case. On the other hand, the methylation will increase the ionisation of the amine polar groups in

alkaline solutions, thus in certain circumstances greatly increasing the interaction of the amine polar group in spite of steric hindrance. Methylation is known to have marked effects on biological activity of certain molecules. This can be due to an easier break-down of the molecule by ferments in its methylated form.

Examples of complex formation between large molecules as measured by the film technique and how they have direct bearing on associations between the molecules in solution give useful analogies as to a possible biological function of the interacting molecules. They give also a good indication of the meaning of the discrepancies found with Overton and Meyer and Traube theories for a series of homologous compounds. For example, at a restricted interface of a long chain alcohol or an alcohol such as cholesterol where the sodium salt of a long chain sulphate is injected into the underlying aqueous phase, an interfacial tension of some 5 dynes/cm. can fall 50 dynes in a minute, although the soap by itself at this concentration (1 mg./250 cc.) will only change the interfacial tension some 10 dynes/cm. over a period of 30 minutes. This change in interfacial tension is also accompanied by great changes in interfacial viscosity. This phenomenon can be shown to be due to the water soluble long chain paraffin ion penetrating into the alcohol monolayer and forming an equimolecular mixed film. This interfacial film on compression collapses as a unit without ejection of either component from the interface. Should the association be broken down by a simple esterification of the alcohol polar group or a *cis*-double bond being formed in the hydrocarbon chain only very weak penetration of the monolayer takes place. The penetrating molecule in this case being easily ejected on compression of the interface back into the aqueous solution or surface lens or crystal. There is in this case only negligible alteration of the interfacial tension. Should, furthermore, the prospective penetrant have two polar groups which are spaced by the hydrocarbon portion of the molecule and are reactive with the interfacial forming polar groups, then no penetration of the interface takes place, but the aqueous soluble molecules adsorb onto the film forming molecules by means of the polar association, and thus form a duplex or double layer. This form of association does not change the interfacial tension, but radically alters interfacial rigidity or elasticity. With large complex molecules both these phenomena occur and examples will be given where slight changes in the chemistry of the compound, an adsorbing molecule can be changed into a penetrating one, with subsequent radical change in biological activity.

## Complexes in Bulk Solution.

For the above-mentioned examples similar radical changes can be shown to exist for the behaviour of the associating compounds composed of hydrocarbon and polar groups both in the aqueous and oil phases.

For example, a long chain hydrocarbon alcohol is insoluble and inactive in water but is readily dispersed into water when mixed equimolecularly with a long chain paraffin ion. This can be shown chemically by inhibition of the precipitation compounds of the soap and biologically by a 100 % increase in the lytic activity of a soap solution by the presence of the long chain alcohol. The reverse of this phenomenon can take place if cholesterol be now substituted for the alcohol ; the lytic activity of the solution being completely inhibited. As can be expected no effects are noticed when the OH polar group is esterified. A further biological analogy is worth noticing here, if bacteria which are unaffected by saponin be incubated with cholesterol lysis of the bacteria takes place. A similar phenomenon takes place if oil is the continuous phase, an oil insoluble long chain paraffin ionic salt is readily brought into dispersion or solution in the oil phase in the presence of an equimolecular concentration of a long chain alcohol or other associating molecule. This solution of a soap or

other poorly soluble oil compound into an oil phase can bring about great changes in three dimensional viscosity.

This system can work even if the associating molecules are the ionised and unionised form of the molecule, for example in half dissociation. Thus oleic acid will readily disperse sodium or calcium oleate into oil.

One may now see how the partition coefficient of a molecule soluble in both phases may be radically altered by the presence of an oil or water soluble interacting or complex forming molecule present at least in equimolecular concentrations. Or even bring into either oil or water solution and thus into biological activity an insoluble molecule by means of complex formation. These concepts must be taken into consideration, when theorising on the Overton-Meyer oil solubility rule for drugs, and possibly explains many puzzling discrepancies found hitherto.

In an even more striking manner can the discrepancies with the Traube theory be clarified. Thus in the previously given example of how the great change in surface tension of an aqueous solution of a long chain paraffin ionic salt can be brought about by the presence of a monolayer of an aliphatic alcohol or cholesterol, which furthermore can be eliminated by small changes in the chemistry or stereochemistry of the alcohols ; then the surface activity of a compound must be made analogous to its drug action or biological action only at the interacting interface. As shown above the interfacial tension can change some 50-60 dynes/cm. for the same compound at interfaces composed of molecules varying only slightly in their chemistry.

It has been shown by Schulman and Rideal that the lytic activity of a series of surface active long chain paraffin ionic salts bear relation only to their surface activity at a cholesterol or protein interface and in no way to their surface tension lowering of an aqueous solution.

## Optimum Activity of a Homologous Series.

It is a general characteristic of a whole range of biologically active compounds that in their homologous series a marked optimum of biological activity is noticed. A series may vary only in the changing number of $(CH_2)$ groups or polar groups, and their mutual spacing or position of a substituting methyl group.

It has been remarked here that the interfacial activity or associating force of a molecule acts on its reactive site in two pronouncedly different ways. The molecules may firstly penetrate an interface forming a mixed interfacial layer or disperse the molecules into their otherwise antagonistic phases, thus causing lysis or enhanced or inhibited activity of the molecule by association, or secondly adsorb on to the interfacial layer, thus tanning the interfacial molecules or causing agglutination of the colloids or cells whose interface has thus become acted upon.

It has been shown by Schulman and Rideal that the optimum of activity of a homologous series can take place at the change over of a molecule from its adsorbing properties on a specific system to its dispersant properties. Thus with the synthetic oestrogenetic compounds *pp'* dihydroxy stilbenes varying only by a $(CH_2)$ the optimum biological activity takes place at the compound's most active adsorbing form on a protein interface, the activity immediately declining again on the molecule showing dispersant action on the interfacial protein molecules. The optimum lytic and hæmolytic activity of a series of surface active compounds varying in number and position of $(CH_2)$ groups and nature and number and mutual spacing of polar groups is at the optimum dispersant action of the molecules on a cholesterol or protein interface. Dr. Hurst will be describing similar optima based on these two conceptions for a homologous series of simple alcohols and acids in their drug action on insects.

Similar analogous optima have been described by Schulman and

Rideal for the cancer-giving hydrocarbons, but in this case it is related to the labile break-down products of the molecules caused by U.V. irradiation in certain organic solutions, and their consequent adsorbtion on to protein interfaces.

Thus the Traube conception of surface activity and the Overton-Meyer conception of oil solubility must be related to the active compounds associating with the specific interacting interface and not generalised for surface activity at an air water interface or oil not in the presence of other oil soluble compounds.

## Molecular Orientation and Biological Activity.

**Lipo-protein Associations.**—It is known that most proteins will spread at air water or oil water interface, or can be made to spread at any aqueous oil interface by suitable dispersion in solvents (alcohols and salts) and suitable charging of the oil interface. It is evident that the protein molecule has undergone some radical change in the process of spreading at the oil water interface, since it is now in an insoluble form. It is presumed that the protein molecule in aqueous solution is a colloid nearly globular in shape, kept in solution or dispersion by means of the ionic and polar groups being orientated into the water phase and the non-polar portions being associated and orientated towards the centre of the colloid. Upon arrival of this molecule at the oil water interface, these various groups in the molecule are separated and reorientated owing to the asymmetric field at the interface.

Thus a protein molecule spread at an interface has its various groups orientated in a triplex layer. The non-polar side chains being pulled into the oil phase and the ionic polar groups pulled into the aqueous phase with the polar keto-imido group in the polypeptide backbone separating the ionic from the non-polar groups. This adlineation of the various groups in the molecule permits them to associate by the short range forces already described either inter- or intra-molecularly.

If these inter-associating groups are small in number per molecule or the molecular weight is relatively small, the protein molecule can be squeezed back into the aqueous solution by means of two-dimensional compression of the interfacial film. With molecules of large molecular weight collapse and fibre formation occur before the molecules can be redispersed. Quite a different picture presents itself if the protein is mixed with lipoids or adsorption of the protein molecule takes place at a lipoid or charged oil water interface, the process now being reversible. The charge being made possible by spreading hydrocarbon ionic compounds, such long chain amines, carboxyls, sulphates, lecithin, kephalin (psychosin, sphingo-myelin) and allied compounds at the oil water interface. Precisely analogous phenomena now occur as described with the salts of hydrocarbon ionic compounds, the protein may adsorb as a double layer on to the lipoid interface or penetrate the interface to form a mixed lipoprotein monolayer, thus greatly changing the interfacial viscosity and rigidity and surface pressure, or the protein molecule may be repelled from the interface by like electrical charges. The great difference between protein and lipo-protein films is that on compression of the mixed film, the protein molecule is ejected from the mono-layer back into the aqueous solution in the form of double layer again, this process being reversible. If now to a lipin-protein mixed film excess lipin be added to the aqueous solution, the protein is completely displaced from the interface and redispersed into the aqueous phase. What form this redispersed protein has taken is of great interest and attempts are being made by use of biologically active molecules to investigate this point.

Frazer, Stewart, Elkes and Schulman have recently shown that most of the phenomena occurring at the oil water interface as described above can

be strikingly demonstrated by means of oil in water emulsions. The oil water interface can be readily charged either negatively or positively by means of negative stabilising agents such as salts of long chain sulphate, carboxylates, and positively by salts of long chain amines and substituted amines. The adsorption of the protein will take place according to the *p*H of the aqueous solution in relation to its isoelectric point, and the sign of the charge on the stabilised oil droplet, together with its zeta potential as controlled by the salt concentration of the aqueous phase. When adsorption takes place the oil droplet is electrically discharged and is surrounded by a rigid or elastic protein monolayer, these neutral droplets can thus agglomerate or agglutinate into bunches and, owing to their density sediment to the top of the aqueous solution. Thus, if protein be added to a stabilised emulsion and the conditions are correct for adsorption, agglutination of the oil droplets takes place with subsequent clarification of the aqueous solution at a protein concentration which is sufficient to cover the surface of the oil droplets with a monolayer. If polyvalent cations or anions be added to the agglutinated emulsion, the agglutination is broken down owing to the removal of the stabilising agent and an oil layer separates out on top of the aqueous solution and the protein is redispersed into the aqueous solution. A similar effect can be achieved by adding excess stabilising agent to the system, the protein coating around the oil droplets being thus deterged away from the interface and protein molecules are redispersed.

The conditions for adsorption of the serum proteins have been demonstrated ; thus serum albumin will adsorb on to positive oil water interfaces above its isoelectric point of *p*H 4·6 and not below this *p*H, and on to negatively charged oil water interfaces at *p*H's below its isoelectric point and not above.

This switch-over from adsorption to no adsorption of the protein with *p*H of the aqueous phase is very sharp at low salt concentrations. Equally, serum globulin will adsorb on to positively charged oil water interfaces above its isoelectric point of *p*H 6·8 and not below and on to negatively charged interfaces below *p*H 6·8 and not above. The sharpness of the change over is less with increasing salt concentration and size or molecular weight of molecule. It is now interesting to note that between *p*H 4·6 and *p*H 6·8 the isoelectric points of the two proteins mentioned, serum albumin will adsorb on to a positive interface and serum globulin on to a negative interface. Thus the proteins can be preferentially adsorbed on to a charged interface from a mixed solution, salt concentration also playing a role in the adsorption.

Frazer and Stewart have shown that when bacteria toxins, snake venom or hormones such as insulin are adsorbed on to emulsions they are not active when injected into animals, but that when the emulsion is broken either *in vivo* or *in vitro* and the protein molecules are redispersed from the oil water interface into the aqueous solution full biological activity returns. This suggests that the protein molecule orientated at the interface is in an inactive form, but can be redispersed into solution and biological activity, when associated with lipoids or lipins.

Likewise, it can be suggested from the first section of this paper that the biological activity of the lipoids and lipins is radically altered when in association with other lipoids, as well as with the proteins.

It can be further suggested that the principles of the short range forces discussed in this paper on the association between lipo-proteins and the influence of other lipoids or chemicals or drugs on these associations will help to clarify some of the actions *in vivo* of drugs.

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# A POSSIBLE MODE OF ACTION OF BENZPYRENE AS A TYPICAL CHEMICAL CARCINOGEN.

By F. Weigert.

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An outstanding feature of the chemical carcinogens is their long delayed action. When the skin of mice is painted with a great excess of the dissolved carcinogen the first tumours do not appear until many weeks, and even months have elapsed. The latent period can be interpreted by biological conceptions, which include the various precancerous states of the tissue until a normal cell is transformed into a malignant cell. We are interested here in a physico-chemical study of the problem whether the carcinogenic drug or one of its metabolites can persist within a living animal over periods which are commensurable with the latent period, and whether it is liable to a spontaneous transformation which may stimulate the change of the cell race. Experiments at the Mount Vernon Hospital have been in progress during the last four years.[1] Benzpyrene was chiefly studied, although it is not the most potent carcinogen, because it is distinguished from other carcinogenic hydrocarbons by a number of different typical fluorescence and absorption spectra.

From the experiments of Peacock, Chalmers and Berenblum and their collaborators [2] it follows that by far the greater part of the benzpyrene which has been introduced into a mouse or rat is changed to non-fluorescent derivatives which have not yet been accounted for. About 1 % is excreted unchanged and the rest appears as blue-fluorescent 'BPX' in the bile and is excreted in the fæces as 5-8-benzpyrene-quinone and as green-fluorescent 'BPF' from which 8-hydroxy-benzpyrene could be isolated.

This metabolism has now been followed in greater detail by fluorescence- and absorption-spectrography and by fluorescence-chromatography which led to the following tentative scheme :

$$\begin{array}{ccccccc} \text{Benzpyrene} & \longrightarrow & \text{`BPX'} & \longrightarrow & \text{`BPF'} & \xrightarrow{(?)} & \text{5-8-Benzpyrene-quinone} \\ & & \downarrow & & \downarrow & & \\ & & \text{[BPX]} & \longrightarrow & \text{[BPF]} & \longrightarrow & \text{8-Hydroxy-benzpyrene} \end{array}$$

[BPX] and [BPF] are prepared by fluorescence-chromatographic purification of extracts from 'BPX' and 'BPF' respectively, from which they are distinguished by their fluorescence spectra.

The blue-fluorescence spectrum of 'BPX' can be seen in the skin, liver, lung and kidney-cortex after application of benzpyrene to a mouse or rabbit. Furthermore, it appears in the bile and small intestine which are the chief route of excretion, and in the milk in the stomachs of suckling mice after intravenous injection of benzpyrene into the mother. Green-fluorescent 'BPF' appears in the alimentary canal beyond the ileo-cæcal valve, in the bladder-urine, and together with 'BPX' in discrete patches of the lung.

[1] Weigert, *Trans. Faraday Soc.*, 1940, **36**, 1033 ; *Nature*, 1942, **150**, 56 ; Weigert and Mottram, *Nature*, 1940, **145**, 895 ; 1942, **150**, 635 ; *Chem. Ind.*, 1941, **60**, 617 ; *Biochem. J.*, 1943, **37**, 497 ; Doniach, Mottram, and Weigert, *Brit. J. Exp. Path.*, 1943, **24**, 1, 9.

[2] Peacock, *Brit. J. Exp. Path.*, 1936, **17**, 164 ; *Amer. J. Cancer*, 1940, **40**, 251 ; Chalmers, *Biochem. J.*, 1938, **32**, 271 ; Chalmers and Crowfoot, *ibid.*, 1941, 35, 1270 ; Berenblum, Crowfoot, Holiday and Schoental, *Cancer Res.*, 1943, **3**, 145, 151.

[BPX] and [BPF] are different with respect to their spectrographic and chromatographic behaviour :

(1) The blue-fluorescence spectra of [BPX] show the same banded structure as adsorbate on alumina and as eluate in alcohol. The fluorescent zone remains fixed at the top of the column even after long development.

(2) The green-fluorescence spectrum of the adsorbate of [BPF] on alumina shows no bands, but the blue-fluorescence spectrum of its eluate in alcohol has a banded structure. The fluorescent zone of [BPF] on alumina moves slowly down during development.

(3) The absorption spectrum of [BPF] in alcohol is very similar to that of 8-hydroxy-benzpyrene, while that of [BPX] is unrelated to any known benzpyrene derivative.

(4) Apart from these chief differences, minor distinctions can be seen according to the origin of the preparations. For instance, the blue-fluorescent zone of [BPX] at the top of the alumina column is narrow and sharp with extracts from bile and small intestine, but extended and diffuse with extracts from liver, while those of kidney-cortex and lung are diffuse but less extended. The diffuse green-fluorescent zones of the adsorbates of [BPF] which move down on development are not so typical, but the fluorescence spectra of their eluates in alcohol show slight displacements of the bands according to the origin of the extracts.

These results show that 'BPX' and [BPX], on the one hand, and 'BPF' and [BPF] on the other hand, comprise two groups, the X- and F-groups respectively, the members of which contain two different benzpyrene derivatives as prosthetic groups combined with various cell constituents. The fluorescence spectra of the adsorbates of the X-group give evidence that the carrier molecule is adsorbed by the alumina, and that the benzpyrene-group is not affected optically by the adsorption. In the F-group the disappearance of the fluorescence bands in the adsorbate indicates that the adsorbed portion of the molecule is the benzpyrene group itself, which is probably identical with 8-hydroxy-benzpyrene.

All members of the X-group are metastable and are readily transformed into members of the F-group. This happens in vivo when bile-'BPX' passes through the ileo-cæcal valve into the cæcum and when kidney-'BPX' enters into the urine. In the lung it occurs apparently in the 'BPX'-holding cells themselves. Post-mortem it can be seen with all 'BPX'-containing tissues, if they are kept for some time at 37°, and even after three days in the ice-chest. This transformation is obviously due to autolysis and to a detachment of the carrier molecules. It can be completely prevented by formalin and other preservatives. [BPX] which is adsorbed on alumina is transformed into [BPF] at elevated temperature in vacuo.

There is no direct evidence whether the 5-8-benzpyrene-quinone is produced directly from 'BPX' or via 'BPF'.

'BPX' appears and is fixed just in those tissues, skin (where it persists in the Malpighian layer for over three weeks), lung and liver, where tumours can be produced by benzpyrene. Hence it is likely that its metastability and its spontaneous transformation into 'BPF' according to the laws of probability may be the reason for the stimulation of the change of a normal into a malignant cell.

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# SOME MORPHOLOGICAL AND OTHER VARIATIONS IN A STRAIN OF BACT. LACTIS AEROGENES ACCOMPANYING ITS ADAPTATION TO CHANGE OF MEDIUM.

By R. M. Lodge and C. N. Hinshelwood.

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Certain relevant conclusions from previous studies of the growth of Bact. lactis aerogenes will first be summarised.

1. The maximum population, $n_S$, which a given medium will support depends partly upon the power of the cells to remove by oxidation inhibitors formed during growth.[1]

2. In an artificial medium consisting of ammonium sulphate, glucose, potassium dihydrogen phosphate and magnesium sulphate (*v.* p. 432), young inocula show a lag phase, referred to as "early lag," during which a growth promoter is formed, diffuses into the medium and stimulates growth of all the cells. This substance is referred to as (L).[2]

3. When Bact. lactis aerogenes which has been grown in bouillon is transferred to the above artificial medium, long snake-like cells, up to 20 times the normal length, are formed if the glucose concentration lies between certain limits. The size distribution of the cells approximately follows the law $n_l = ne^{-l/\bar{l}}$ where $n_l$ is the number of cells of length greater than $l$, $n$ the total number and $\bar{l}$ the mean length. The interpretation given is that we are dealing with a condition where division is delayed: the probability of division is here the limiting factor, whereas normally the elongation of the cell is rate determining.[3]

A size coefficient, $\sigma$, is defined by the relation $\sigma = \sum_{l=3}^{l=\infty} l \cdot \nu_l$ where $\nu_l$ is the number of cells in unit range in the neighbourhood of $l$ (measured in certain arbitrary units). $\sigma$ gives a good representation of the abnormal or "snake-like" appearance of the culture. During the growth cycle it rises to a maximum and then falls, often to zero.

The influence of various factors, including filtrates from older cultures, on $\sigma$ leads to the hypothesis that there are two independent factors referred to as (L) and (D) which control elongation and division of the cells respectively.

With successive subcultures of the organism $\sigma$ varies in such a way as to suggest that the enzyme systems responsible for the (L) and (D) factors are easily thrown out of balance, and that by a slow process of adaptation they can be brought into balance again. A theory of this adaptation is given by Hinshelwood and Lodge in the paper referred to.

## Bact. Lactis Aerogenes (Morris).

This was a strain obtained from the National Collection of Type Cultures (Number 5268) and differed from that used in all previous work in that it had, in the glucose-ammonium sulphate medium, a much slower growth rate (m.g.t. of about 120 minutes, compared with the normal 32 minutes), and at first grew with a long lag to a small value of $n_s$.

On repeated subculture in artificial media, after transfer from bouillon (heart broth), it showed remarkable phenomena of adaptation. $n_s$ pro-

[1] Lodge and Hinshelwood, *J.C.S.*, 1943, 208. [2] *Ibid.*, 214.
[3] Hinshelwood and Lodge, 1943, *P.R.S., B*, *in the press*.

gressively increased to many times its initial value and the (D) and (L) producing functions first of all became increasingly unbalanced—as shown by the production of long snake-like cells—and later came into normal relation once more—as shown by the elimination of these snake-forms.

We conclude from this that the three functions of the cell, (*a*) the oxidation of inhibitors (*v.* para. 1, above), (*b*) the synthesis of the lag remover, (L) (*v.* para. 2, above) and (*c*) division, are all adapting themselves to the new medium at separate and independent rates.

It follows that the three functions which are here revealed as independent should themselves be susceptible to separate control, *e.g.*, by drug action. Several kinds of observation can be correlated from this point of view. For example, (*a*) $p$H can affect $n_s$ without affecting m.g.t.[4] or lag,* (*b*) lag can be prolonged or reduced independently of m.g.t. or $n_s$,[5] (*c*) snake-like forms can be induced by chemical agencies [6] in such a way as to indicate that the division function is specifically impaired.

In what follows, the behaviour of the strain "Morris" of Bact. lactis aerogenes leading to this conclusion is described.

## A. Serial Subcultures in Artificial Media.

Bacteria were transferred from a stock bouillon culture to aerated artificial media, and subcultures, using 1·0 cc. inocula for 26·0 cc. of fresh artificial medium, were made every 48 hours. The media contained as nitrogen source ammonium sulphate, the equivalent amount of an amino acid (asparagine or alanine), or a mixture of ammonium sulphate and an amino acid. The mixture was originally used because growth in ammonium sulphate was difficult, and it was thought that the cells could be trained to use it by a gradual reduction to zero of the amount of amino acid in a mixture with ammonium sulphate. The course of training, however, proved to be more complex than expected.

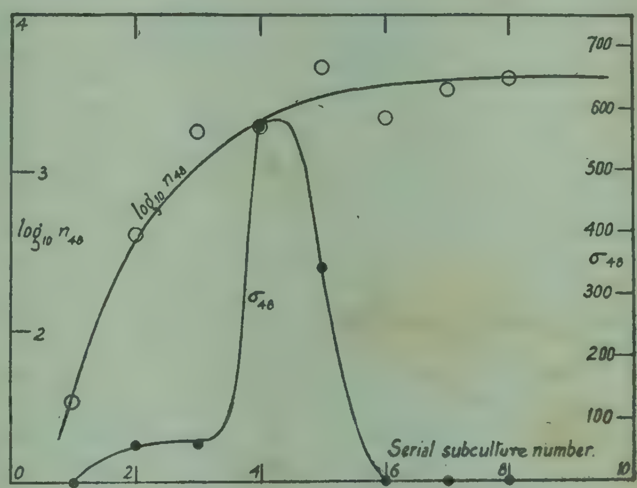


FIG. 1.

Fig. 1, for a typical series of subcultures in a medium containing a constant mixture of ammonium sulphate and asparagine as nitrogen source, shows the course of the adaptation from bouillon to this medium. (Here, $n_{48}$ refers to haemocytometer counts of samples taken 48 hours after inoculation. The value of $n_{48}$ does not differ significantly from that of $n_s$ in the same culture. Values of $\sigma_{48}$ greater than about 20 indicate that a culture has a definitely abnormal appearance—*v.* para. 3 above.)

It should be noted that $n_{48}$, the logarithm of which is plotted in Fig. 1, starts at a small value and increases steadily to a limit as the serial subculture number is increased. Upon subculture for the fourth time, when the maximum population has attained almost to the limit, $\sigma_{48}$ rises sharply

[4] Lodge and Hinshelwood, *J.C.S.*, 1939, 1683.

* Unpublished observation.

[5] *Cf.* Lodge and Hinshelwood, *J.C.S.*, 1939, 1692.

[6] *Cf.* p. 428, and also Hinshelwood and Lodge, ref. 3.

to the high value of 580 and the culture shows a remarkable appearance. It is clear that the division function has become quite out of balance with the elongation of the cells at this stage ; this is, according to our hypothesis, due to the development of a corresponding lack of balance between the (L) and (D) factors. The balance is nearly restored after the sixth subculture, though long cells occur sporadically during many subsequent ones.

There has been a development of the power of the cells to multiply to a high $n_{48}$ : there has also been development of their capacity to elongate and to divide in the new media. All these processes seem to have evolved independently and at different rates.

Qualitatively similar behaviour is shown by successive cultures of the organism in media containing as nitrogen source a mixture of ammonium sulphate and alanine, asparagine alone, or ammonium sulphate alone (Table I). At some stage in a series, after $n_{48}$ has become more or less

TABLE I.

| Serial Subculture Number. | Ammonium Sulphate. | | Asparagine. | | Ammonium Sulphate + Asparagine. | | Ammonium Sulphate + Alanine. | |
|---|---|---|---|---|---|---|---|---|
| | $n_{48}$. | $\sigma_{48}$. | $n_{48}$. | $\sigma_{48}$. | $n_{48}$. | $\sigma_{48}$. | $n_{48}$. | $\sigma_{48}$. |
| 1 | 188 | 0 | 36 | 0 | 33 | 0 | 108 | 0 |
| 2 | Tangle | | 80* | 0 | 400* | 60 | 18 | 0 |
| 3 | Tangle | | Tangle | | 1900 | 60 | 256 | 0 |
| 4 | Tangle | | 1200 | 580 | 3000 | 580 | 700 | 200 |
| 5 | 2760 | 870 | 1500 | 25 | 4800* | 350 | 480 | 200 |
| 6 | 3500 | 50 | 2600 | 15 | 2200 | 0 | 800 | 0 |
| 7 | 4000 | 100 | 1400 | 48 | 3600 | 0 | 360 | 10 |
| 8 | 4500 | 30 | 1000 | 27 | 4000 | 0 | 340 | 28 |

* Transfer from these cultures into ammonium sulphate media gave tangles.

stabilised, cultures begin to assume a thread-like appearance which, on further subculture, eventually disappears.

Sometimes, at certain stages in a series, the cells clump together to such an extent that counting becomes impossible. This must depend upon another function which seems to develop independently, but, from its nature, it does not lend itself to quantitative measurement.

If, in a given series of subcultures, the inoculum size is reduced to a tenth, at a stage when adaptation is just complete (as shown by stabilised behaviour in successive subcultures) a certain regression occurs and snake-like forms re-appear. Sudden reduction of the inoculum size can thus cause an upset of the enzyme balance, which must depend upon indirect effects—changes in lag (*v.* para. 2, above), concentration of growth promoters in the medium, and so on.

## B. Transfer from Media with Amino Acid as Nitrogen Source to Media with Ammonium Sulphate.

When Bact. lactis aerogenes (Morris) is transferred from bouillon to artificial media, there is a drop in $n_{48}$ from 800 to about 100. On further subculture in the artificial medium, $n_{48}$ reaches several thousands. There can, however, be a much more striking change when the organisms are transferred from a medium containing an amino acid nitrogen source to one containing ammonium sulphate.

If a culture grown several times in media containing amino acid mixed with ammonium sulphate is transferred to one with ammonium sulphate alone, growth in the latter appears under the microscope as a tangled skein of threads of almost indefinite length. The culture is remarkable for the

complete absence of cells of normal size; the division probability seems to be practically zero. A second subculture in ammonium sulphate may show this phenomenon even more markedly, but, on continued cultivation in ammonium sulphate, the cells tend to become normal once more, although some very long cells may still appear after eleven subcultures (*v*. C, below).

It would seem that, in the media containing a mixture of asparagine or alanine with ammonium sulphate, the bacteria become adapted to use the amino acid rather than the ammonium sulphate. We already have evidence (Hinshelwood and Lodge) [3] that (D) is formed easily in amino acid media; in such media there will be little development, therefore, of the mechanism by which (D) is produced in ammonium sulphate media. Accordingly, when the cells are deprived of amino acid, the (D)-forming function may be very deficient, and adaptation to growth on ammonium sulphate must occur. The transient derangement of the enzyme balance is at first so great that it leads to the appearance of the tangled threads.

The phenomenon is not easy to reproduce. It can occur as well with the third as with the fifteenth subculture in the mixture on transfer to ammonium sulphate, but it varies somewhat erratically with the age of the culture before transfer.

### C. Size Distribution of Cells.

Reference was made in paragraph 3 to the logarithmic size distribution of the cells of the ordinary fast-growing strain of Bact. lactis aerogenes grown in the artificial medium at certain glucose concentrations.

TABLE II.—NITROGEN SOURCE OF INOCULANT: AMMONIUM SULPHATE + ASPARAGINE. INOCULATION INTO AMMONIUM SULPHATE MEDIUM.

| Size Range. | Number of Cells in the Range. | |
|---|---|---|
| $\frac{1}{2}$ | 130 | Rapid decline, probably following logarithmic law. |
| 1 | 132 | |
| 2 | 72 | |
| 3 | 26 | |
| 4 | 0 | |
| 5–10 | 37 | Secondary distribution with a different law. |
| 10–15 | 36 | |
| 15–20 | 16 | |
| 20–25 | 16 | |
| 25–30 | 3 | |
| 30–40 | 3 | |

TABLE III.—DECLINE OF THE SNAKE-LIKE FORMS ON SERIAL SUBCULTURE IN AMMONIUM SULPHATE OF THE CULTURE ANALYSED IN TABLE II.

| Serial subculture number:— | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Size Range. | % Bacterial Substance in the Size Range. | | | |
| 0–2 | 18·2 | — | 60·6 | 70·5 |
| 2–5 | 10·0 | — | 21·2 | 17·4 |
| 5–10 | 13·5 | — | 10·4 | 9·6 |
| 10–25 | 48·4 | — | 7·8 | 2·5 |
| 25–40 | 9·9 | — | 0 | 0 |

Generally similar distributions are shown by the "Morris" strain, except when the tangle of threads appears. The size distribution is then different. Over the range of smaller sizes ($l = \frac{1}{2}$ to 4) the logarithmic law is approximately followed. There is, however, in the larger size range ($l = 5$ to 40) a secondary distribution with a different law.

Table II gives numbers showing this for a culture growing in ammonium sulphate and inoculated from an asparagine-ammonium sulphate mixture. When grown, the transferred culture contained many long cells, but was not a tangle of the most marked kind. On further subculture in ammonium sulphate, the frequency maximum receded to the smaller size ranges, as shown in Table III.

It is not proposed to discuss the size distribution in detail. It will only be remarked that the distribution in the longest size ranges may be

determined more by the mechanical snapping of threads which might have reached an indefinite length than by the division probability, which may well approach zero.

**Summary.**

When a certain (slow-growing) strain of Bact. lactis aerogenes is transferred from a given medium to one of a different kind and is subcultured many times in the latter, the stationary populations attained and the morphological variations occurring in successive subcultures indicate that the individual cell functions responsible for (*a*) removal of growth inhibitors, (*b*) elongation and (*c*) division are all undergoing adaptation independently and at different rates.

In certain circumstances (*b*) and (*c*) may become so unbalanced that thread-like cells of enormous length are formed.

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# THE EFFECTS OF RESORCINOL AND OF *m*-CRESOL ON THE GROWTH OF BACT. LACTIS AEROGENES.

BY G. H. SPRAY AND R. M. LODGE.

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A study of the influences of disinfectants on bacteria may be expected to shed light on the numerous chemical reactions involved in life processes. To this end, the effects of resorcinol and *m*-cresol, as part of a series of phenols, on the growth of Bact. lactis aerogenes in a liquid artificial medium have been investigated. It has been found that the duration of bacterial lag, the growth rate and the stationary population to which the culture attains are all influenced by the presence of disinfectant. The precise relationships between each of these quantities are not, however, identical, indicating that these disinfectants act by retarding particular cell reactions rather than by exerting a general depressant effect on metabolism.

Bacterial lag varies with the age of the parent culture. With the particular organism and medium used in this work it has been shown [1] that there is a considerable lag (known as " early lag ") when the inoculant itself has just started to grow. As growth of the inoculant proceeds, the lag first falls to a minimum and then increases (" late lag ") on further ageing of the inoculant. The variation of early, minimum and late lags with disinfectant concentration has been studied.

The rate of bacterial growth is inversely proportional to the mean generation time (m.g.t.), which is the time taken for the population to double. Results are expressed as the ratio of the value of the m.g.t. for a culture containing no disinfectant to that obtained in the presence of disinfectant. Stationary population ($n_s$) is defined as the count at the time when the growth rate falls to a low value and the culture enters the stationary phase.

**Experimental.**—The medium consisted in an aqueous solution of glucose (35·7 g./l.), potassium dihydrogen phosphate buffer (3·21 g./l.) with enough sodium hydroxide to bring the $p$H to 7·12, ammonium sulphate (0·89 g./l.), magnesium sulphate ($3·57 \times 10^{-2}$ g./l.), together with the

[1] Lodge and Hinshelwood, *J.C.S.*, 1943, 213.

desired amount of disinfectant. The water used was distilled from the laboratory supply of distilled water through a silica condenser. Media, contained in sterile pyrex culture tubes suspended in a water thermostat at 40·0°, were aerated with a slow stream of washed sterile air. They were inoculated, sampled and counted by methods described previously.[2] The bacteria were stored in a meat extract medium and all inocula for the experiments to be described were derived from this after two passages through the aerated medium with no added disinfectant.

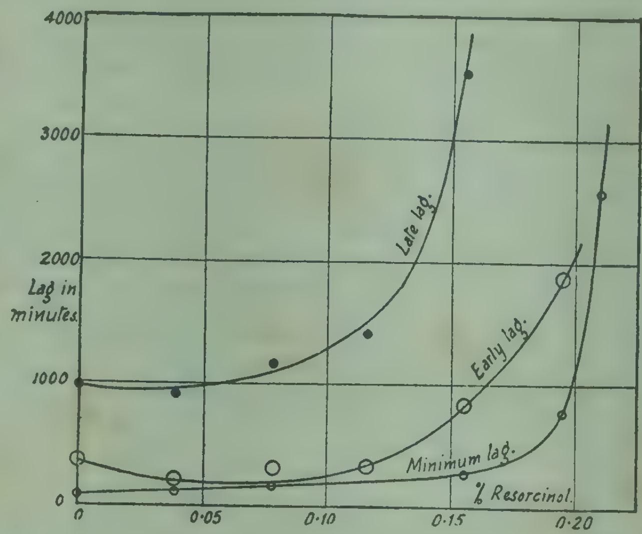


FIG. 1.

## A. Resorcinol.

**Lag.**—The results are given in Fig. 1. They show that (i) small concentrations of resorcinol reduce early and late lags slightly, (ii) at higher concentrations the lag increases rapidly, and (iii) the limiting resorcinol concentration, above which lag becomes infinite, depends upon the age of the inoculant.

Reduction of early and late lags implies that resorcinol either reacts with one of the constituents of the medium to give a substance capable of shortening the lag, or that it inhibits the action of some substance which lengthens lag and which is present as unavoidable impurity in the materials of which the medium is composed. The former hypothesis is supported by the observation that a yellow fluorescence develops when sterile media containing resorcinol are aerated at 40° for several days. The fluorescence depends for its formation on the presence of both glucose and phosphate, suggesting that resorcinol forms a compound with glucose * in the presence of the buffer.

TABLE I.—THE EFFECT OF RESORCINOL ON STATIONARY POPULATION.

| Resorcinol Concn., %. | Average Value of Stationary Population. | Number of Determinations. |
|---|---|---|
| 0·000 | 1972 | 3 |
| 0·020 | 1514 | 1 |
| 0·039 | 1176 | 12 |
| 0·058 | 1202 | 1 |
| 0·078 | 1193 | 11 |
| 0·097 | 1479 | 1 |
| 0·116 | 1039 | 4 |
| 0·155 | 1093 | 3 |
| 0·195 | 789 | 3 |
| 0·210 | 750 | 1 |

**Stationary Population.**—The stationary population is independent of resorcinol concentration within the range which permits growth (Table I). This indicates that resorcinol is destroyed or titrated by the bacteria during

[2] Dagley and Hinshelwood, *J.C.S.*, 1938, 1930.
* *Cf.* Snell and Snell, *Colorimetric Methods of Analysis* (*Organic and Biological*), 1937, p. 470.

growth. By the time the stationary phase is reached, no resorcinol is left in the medium, and the final population is limited by other factors.[3]

**Growth Rate.**—Fig. 2 shows the variation in growth rate with resorcinol concentration for inocula of different ages. It will be seen that, when the inoculum is such that lag in the fresh medium is at a minimum, the growth rate is independent of resorcinol concentration up to about 0·08 %. Above this, the growth rate falls linearly with increasing resorcinol concentration. This type of behaviour is not given by either older or younger inocula. In these cases, as the resorcinol concentration is increased, the growth rate falls at first, then rises to about its original value, finally falling linearly as before.

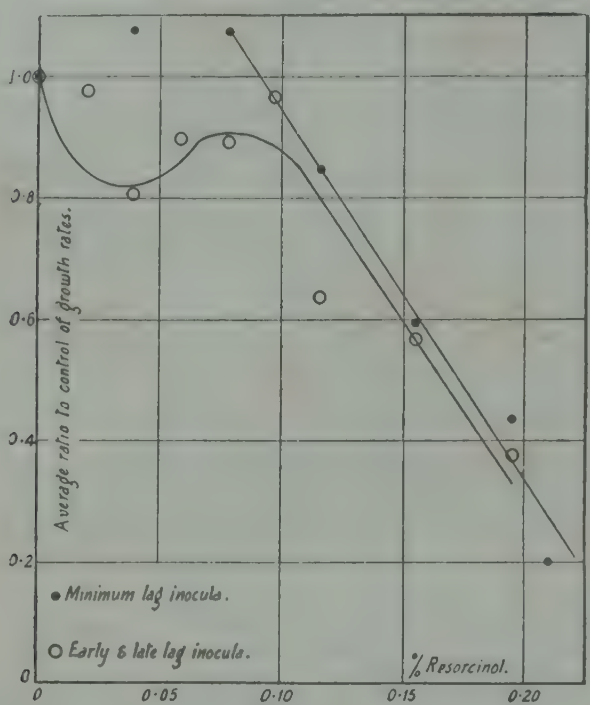


Fig. 2.

The constancy of stationary population suggests destruction or inactivation of the resorcinol. The way in which the growth rate varies with resorcinol concentration may be explained on the same basis. With both very young or very old inocula, the growth rate is *less* at 0·04 % resorcinol than it is at 0·08 %. However, at the higher resorcinol concentration the lag is longer (Fig. 1); accordingly, at the higher concentration, more time is available for the resorcinol to be destroyed by the bacteria, and, at the start of growth, the effective resorcinol concentration is greater in a medium initially containing 0·04 % resorcinol than in one containing 0·08 %. At concentrations greater than about 0·08 %, more resorcinol is present than the bacteria can neutralise during the lag phase, and the growth rate falls with increasing disinfectant concentration in the usual way.

TABLE II.—The Effect of the Age of an Inoculant free from Disinfectant on the Growth Rate in a Medium containing 0·078 % Resorcinol.

| Age of the Inoculant, Minutes. | Growth Rate, $k \times 10^2$. |
|---|---|
| 253 | 1·63 |
| 372 | 1·66 |
| 434 | 2·00 |
| 491 | 1·89 |
| 583 | 2·14 |
| 677 | 1·89 |
| 2204 | 1·82 |

Curves obtained by plotting the logarithms of the total bacterial counts against time are normally straight lines, the population, $n$, increasing with time, $t$, approximately according to the equation $n = n_0 e^{k(t-L)}$, where $n_0$ is the initial number of bacteria, $L$ the length of the lag phase, and $k$ the growth rate constant ($= \log_e 2$/m.g.t.). Resorcinol cultures derived from inocula at the stage of minimum lag conform closely to this, but when younger or older inocula are used, the initial stages of such growth curves

[3] *E.g.* Graham-Smith, *J. Hyg.*, 1920, **19**, 139; Lodge and Hinshelwood, *J.C.S.*, 1939, 1683.

are usually concave to the long $n$ axis. The value of $k$ increases as growth proceeds, indicating that the effective resorcinol concentration is falling during this time. Inocula taken from cultures at the minimum lag stage themselves appear to be able to destroy considerable amounts of resorcinol. Furthermore, there is in these cases a definite initial tolerance to resorcinol with respect to both lag and growth rate.[4] It appears, therefore, that the bacteria synthesise some substance capable of neutralising the effects of resorcinol, and that this substance is present in greatest amount at the stage of minimum lag.

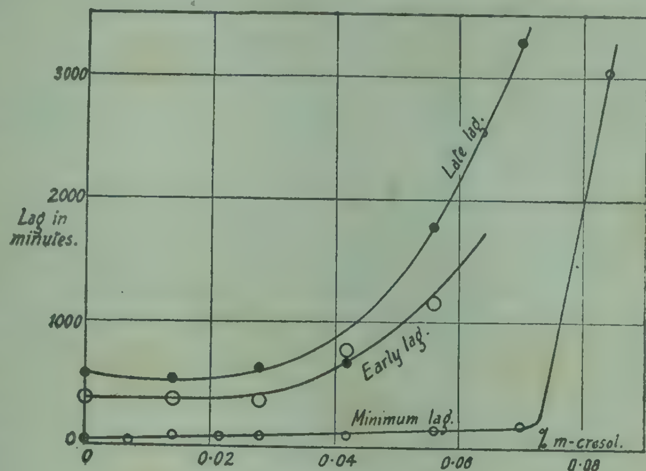


FIG. 3.

To investigate this further, the variation in bacterial growth rate in the presence of 0·0775 % resorcinol with the age of a resorcinol-free inoculant has been determined. The results, given in Table II, confirm that the concentration of the resorcinol inhibitor passes through a maximum at a time corresponding roughly to the stage of minimum lag.

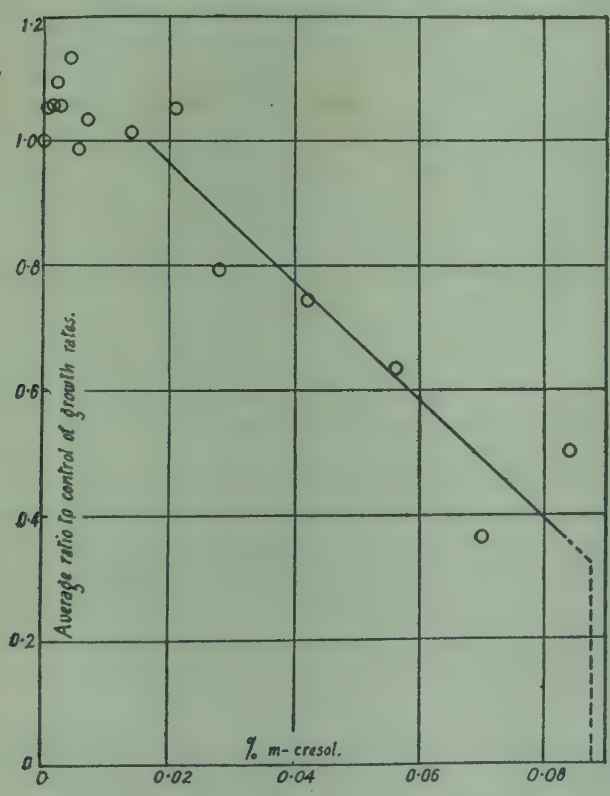


FIG. 4.

The inhibitor may be intracellular or it may be excreted by the bacteria into the medium. In the latter case, the addition of filtrate from a culture in the stage of minimum lag to media inoculated from a similar culture should increase the tolerance range of the bacteria to resorcinol. No such effect could be observed, and it may be concluded that the substance remains in the cells.

## B. *m*-Cresol.

**Lag.**—The effects of *m*-cresol on the lag of Bact. lactis aerogenes in the artificial medium are generally similar to those produced by resorcinol (Fig. 3). A similar reduction of early and late lags by small concentrations of disinfectant is observed. The highest concentration at which growth can occur is 0·084 % *m*-cresol with inocula

[4] *Cf.* Poole and Hinshelwood, *J.C.S.*, 1940, 1565.

taken at the stage of minimum lag. With early and late lag inocula the limit is about 0·06 %.

**Growth Rate.**—There is an appreciable initial tolerance up to a limit at 0·02 % *m*-cresol. Increase of concentration above this value leads to a linear decrease in growth rate (Fig. 4). Very small amounts of *m*-cresol appear to cause a slight increase in growth rate.[5] This is shown not to be due to the bacteria utilising *m*-cresol as a source of carbon more readily than glucose, since no growth could be detected in media free from glucose, but containing small amounts of *m*-cresol. The *m*-cresol may possibly react with some constituent of the medium so as to bring the latter into a more readily available form.

**Stationary Population.**—Table III. shows that there is a slight fall in the stationary population as the concentration of *m*-cresol is increased. This fact, together with the tolerance of the organism to *m*-cresol with respect to growth rate, indicates that the disinfectant is partially neutralised during the growth of the bacteria. This corresponds to type 3*a* of the growth rate-disinfectant concentration curves classified by Poole and Hinshelwood.[4]

TABLE III.—THE EFFECT OF *m*-CRESOL ON STATIONARY POPULATION.

| *m*-Cresol Concn., %. | Average Stationary Population. | Number of Determinations. |
|---|---|---|
| 0·000 | 1262 | 5 |
| 0·0007 | 966 | 1 |
| 0·0014 | 1175 | 1 |
| 0·0021 | 1862 | 1 |
| 0·0028 | 1585 | 1 |
| 0·0042 | 1084 | 1 |
| 0·0056 | 2042 | 1 |
| 0·0070 | 1334 | 2 |
| 0·014 | 918 | 3 |
| 0·021 | 785 | 1 |
| 0·028 | 918 | 3 |
| 0·042 | 811 | 3 |
| 0·056 | 547 | 3 |
| 0·070 | 546 | 4 |
| 0·084 | 661 | 1 |

At the highest concentration at which growth can be observed (0·08 %), the stationary population is about 500; this is to be compared with 1500 for a culture containing no disinfectant. In this case, therefore, factors other than the reduction to zero of the stationary population preclude growth at higher disinfectant concentrations. Near the limiting *m*-cresol concentration, the lag is increasing very rapidly, but the growth rate is only falling slowly. It is clear that the increase to infinity of the lag limits growth under these conditions. Similarly, the limiting concentration of resorcinol is that at which the lag is tending to become infinite, whereas, in the case of phenol[4] the controlling factor is the reduction of the growth rate to zero.

**Morphological Changes Induced by *m*-Cresol.**—When the particular strain of Bact. lactis aerogenes used in this work is grown in media containing 0·07 % *m*-cresol, it undergoes a striking morphological change when the conditions are suitably adjusted. The bacteria, instead of appearing as the normal rod-shaped cells of length about 1 $\mu$, undergo elongation along one axis to give thread-like cells. Single cells up to many hundreds of times the normal length are formed, and they may grow to lengths as great as 0·25 mm.

The production of filaments has been observed with Bact. typhosum when grown in the presence of methyl violet,[6] with Streptococcus viridans in the presence of sulphanilamide,[7] and with Clostridium welchii, the cholera vibrio and numerous other organisms in the presence of concentrations of penicillin insufficient to inhibit growth completely.[8]

[5] *Cf.* Salter, *J. Inf. Dis.*, 1919, **24**, 260.
[6] Ainley Walker and Murray, *Brit. Med. J.*, 1904, **2**, 16.
[7] Tunnicliff, *J. Inf. Dis.*, 1939, **64**, 59.
[8] Gardner, *Nature*, 1940, **146**, 837.

Similar morphological changes occurred when the organisms were cultured in media in which the glucose concentration had been reduced to a twentieth of its normal value.[9] Here, the distribution of sizes of the cells was governed by the relation $n_l = ne^{-l/\bar{l}}$, where $n_l$ is the number of cells with lengths greater than $l$, and $\bar{l}$ is the mean length of all the cells of total number $n$.

To explain the phenomena occurring in dilute glucose media, it was suggested that the bacteria synthesise an intracellular substance (D) which influences their division. Evidence was adduced that another substance (L), synthesised and excreted by the bacteria, must be present in concentrations greater than a critical value before growth of bacterial substance, as distinct from division, can occur. In a similar way, it was assumed that (D) must be present in more than a critical concentration before division can occur. Filaments are formed when elongation proceeds normally while the probability of division is low, *e.g.*, when the concentration of (D) is below the critical.

In media containing 0·07 % *m*-cresol, the value of $\bar{l}$ is very much greater than in the dilute glucose media. The actual number of cells is proportionately smaller, and a statistical study of sizes becomes more difficult. Fewer bacteria are in the field of view of the microscope at any one time, and the sizes of the very long cells are difficult to assess, since they tend to become tangled.

Since the filaments are very much longer in cultures containing *m*-cresol under the conditions described below, it appears that (D) is in very short supply, and that its synthesis is slowed down by *m*-cresol.

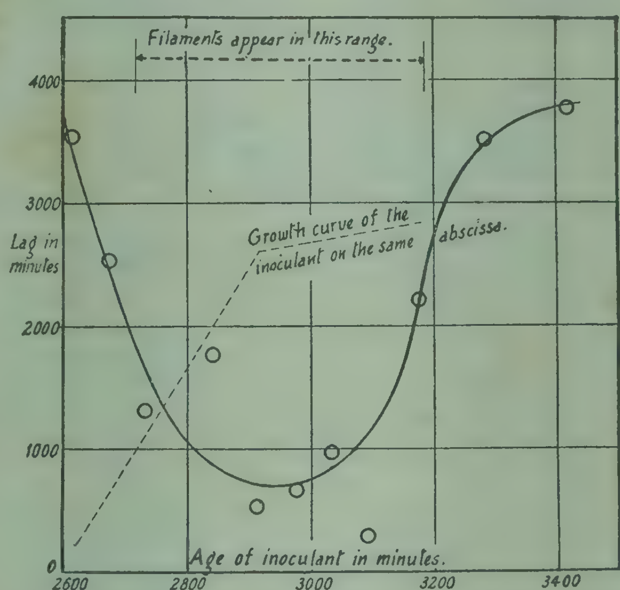


Fig. 5.

**Conditions for the Formation of Snake-like Cells by *m*-Cresol.** We may now examine the conditions under which filaments are produced in the presence of *m*-cresol, and how each case may be explained by the above working hypothesis.

If a standard culture is subcultured at the minimum lag stage into a medium containing 0·07 % *m*-cresol, filaments are produced after growth in the new medium has proceeded for some time. The filaments usually tend to disappear in the later stages of the culture cycle. The rate of synthesis of (D) is thought to be cut down by *m*-cresol, so that, as growth in the new medium proceeds, the concentration of (D) in the cells is not maintained at its original level in the inoculum; when it falls below the critical value the cells elongate but do not divide.

Minimum lag inocula containing 0·07 % *m*-cresol give enhanced filament production when subcultured in the presence of 0·07 % *m*-cresol. In this case the inoculum itself contains snake-like forms, and may be supposed to be lacking in (D). Growth in the presence of *m*-cresol therefore starts under conditions favourable for the production of filaments. Hence,

[9] Hinshelwood and Lodge, 1943, *P.R.S., B, in the press.*

elongation proceeds for a longer time than in the former case, and increased production of snake-like forms results.

If, instead of subculturing into media containing *m*-cresol, inocula similar to the above are grown in the standard medium free from disinfectant, no snake-like forms appear. Under these conditions, the rate of synthesis of (D) is always great enough for it to be provided in adequate amounts. As before, the inoculum from the culture containing *m*-cresol is deficient in (D). Even though lag is at its minimum, it is long enough for the deficiency to be made good before growth starts. The bacteria then divide as soon as other conditions become favourable, and the culture accordingly shows a normal appearance. The same reasoning applies to inocula taken at the late lag stage from media containing no *m*-cresol.

Inocula taken from cultures with or without 0·07 % *m*-cresol at the late lag stage do not produce filaments in media containing 0·07 % *m*-cresol. It appears, therefore, that the lag is long enough for the bacteria to build up a concentration of (D) so great that it is maintained above its critical value throughout the period of elongation. The reserve so formed is sufficiently great even though the synthesis of (D) is slow.

After the organisms have been subcultured ten times into media containing *m*-cresol, no diminution in the production of filaments is evident provided that all inocula are taken at the minimum lag stage. Thus, no adaptation of the bacteria to the disinfectant occurs.

TABLE IV.—The Effect of the Age of an Inoculant Culture containing 0·07 % *m*-Cresol on the m.g.t. of Subcultures into Media free from Disinfectant.

| Age of the Inoculant, Minutes. | m.g.t., Minutes. |
|---|---|
| 940 | 47·6 |
| 1041 | 45·8 |
| 1176 | 40·6 |
| 1401 | 98·0 |
| 1640 | 53·6 |
| 2397 | 54·0 |
| 3180 | 30·4 |

When a culture containing 0·07 % *m*-cresol is put into media containing the same concentration of disinfectant, the lag falls to a minimum at the onset of the stationary phase of the inoculant,[10] and rises as the stationary phase proceeds (Fig. 5); filament production tends to a maximum when lag is shortest, no snake-like forms being given by either very young or very old inocula. With such inocula a sufficient reserve of (D) is built up before growth begins, so that it is not exhausted while elongation is in progress. When the lag is short enough, there is a lack of (D) at some point in the growth cycle and filaments are produced.

If a culture containing 0·07 % *m*-cresol is put back into the medium free from disinfectant at the onset of the stationary phase, the growth rate of the subculture has a normal value. As the inoculant ages further, the growth rate falls to a minimum and filaments appear only in those subcultures which grow slowly. Maximum production of snake-like cells coincides with slowest growth. As the inoculant ages still further, the growth rates of the subcultures gradually revert to normal and the production of filaments ceases simultaneously (Table IV). If the slow-growing organisms are repeatedly subcultured in media containing no *m*-cresol, their growth rates gradually increase and the tendency to give filaments gradually disappears. Almost complete recovery is brought about by forty such subcultures, but it is evident that the cells must have suffered some profound change for recovery to take so long. This change is further evidenced by the variation in growth rate with the age of the inoculant culture, described above.

To explain these phenomena it is suggested that, during the stationary phase as the inoculant containing *m*-cresol ages, a point is reached at which

[10] *Cf.* Lodge and Hinshelwood, *J.C.S.*, 1943, 213.

the permeability of the cell walls to *m*-cresol falls abruptly and almost to zero. It must also be assumed that the *m*-cresol present in the cells modifies both those parts of the bacteria responsible for synthesis of (D) and those which control the growth of bacterial substance, and that the change takes a considerable time to occur. The modification in fact occurs mainly during the lag phase between inoculation and the start of growth in the medium free from *m*-cresol.

Thus, when a culture containing 0·07 % *m*-cresol is subcultured in the early part of the stationary phase, the lag is short and there is little time for any damage to occur. Hence, normal growth ensues. With older inocula, the lags of the subcultures become longer and there is more time for the *m*-cresol to act. Hence, growth is slow and filaments are formed. Finally, however, the state is reached when the *m*-cresol can no longer penetrate the cell walls, and that which is already inside the cells must become neutralised by oxidation or other means. Upon subculture at this stage, therefore, the cells suffer no damage and growth is again normal.

It is a pleasure to record our gratitude to Professor Hinshelwood for his help in connection with this work.

## Summary.

The influences of resorcinol and of *m*-cresol on lags, growth rates and stationary populations of Bact. lactis aerogenes in liquid artificial media have been studied.

The ways in which these characteristics of growth are changed by various concentrations of disinfectant lead to the following conclusions.

(1) Both disinfectants have specific actions on various stages of cellular metabolism in different ways.

(2) Indefinite prolongation of the lag rather than reduction of the growth rate or of the stationary population to zero causes inhibition of growth at the higher disinfectant concentrations.

(3) The effects of resorcinol can be completely neutralised by the bacteria during growth; with *m*-cresol, on the other hand, only partial neutralisation occurs.

At a certain concentration of *m*-cresol, and with carefully controlled inocula, the bacteria undergo a morphological change and grow to thread-like cells many times their normal length. The theory that elongation and division are processes controlled by separate factors is applied to the experimental results.

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# THE ADAPTATION OF BACT. LACTIS AEROGENES TO GROWTH IN THE PRESENCE OF SULPHONAMIDES.

By D. S. Davies and C. N. Hinshelwood.

*Received 22nd July,* 1943.

The object of the work to be described was to study the action of sulphonamide compounds upon the growth of Bact. lactis aerogenes. Previous work with this organism showed that various antiseptic agents acted in specific ways upon different parts of the growth mechanism, some, for example, lengthening the lag phase, others reducing the actual rate of

multiplication progressively to zero.[1] The action of the typical sulphonamides chosen proves to be unlike that of most other antiseptic agents: it is never complete, and it provokes responses whereby the bacteria become immune to the drug. The immunisation—well known in the clinical literature—is itself of some complexity. Some of the threads of these matters have now been disentangled. The results may be of some indirect medical interest, but we shall be primarily concerned with the help they may give towards constructing a physico-chemical theory of cell growth and adaptation.

**Method.**—The strain of Bact. lactis aerogenes used had been maintained since 1937 by monthly subculture in bouillon. Minor changes in growth rate were observed between 1937 and 1942, but experiments in 1942 showed that the strain was still homogeneous.

An artificial medium was used in all experiments. It contained: 10 cc. of glucose 100 g./l.; 10 cc. $KH_2PO_4$, 9 g./l., *p*H adjusted to 7·2 with NaOH; 5 cc. of either a 5 g./l. solution of ammonium sulphate or of a 5 g./l. solution of asparagine; 1 cc. magnesium sulphate, 1 g./l. Solutions were made up in twice distilled sterile water and sterilised by intermittent boiling.

The sulphonamides in aqueous solution were added as required, the additions not normally exceeding 2 cc. To obtain higher concentrations of sulphaguanidine (which is less soluble than sulphanilamide) portions of a solution saturated at 50° were added.

The inhibition of growth, known from the original work of Tréfouel and others,[2, 3] was followed by examination of the complete growth curves, which were determined as follows. A tube of the standard medium was inoculated with a loop or two of the bouillon culture and incubated at 39·7° in a gentle stream of sterile air. At a suitable stage in its growth this culture was used as inoculant for quantitative experiments. Inocula were transferred by means of a 0·1 cc. pipette: the initial populations varied from $10^6$ to $10^7$ cells per cc. When cloudiness began to develop, samples were withdrawn at suitable intervals, killed and stained immediately, and set aside for counting. Counts were determined in a haemocytometer with a chamber of depth 0·02 mm. The mean count, $n$, is given per unit square: the population per cc. is $1{\cdot}25 \times 10^6\, n$.

**Characteristics of the Normal Growth of Bact. lactis aerogenes in the Standard Medium.**—In normal growth there is a well-defined logarithmic phase, where $n = n_0 \exp. (kt)$. This usually continues to $n = 800$, when growth slows up sharply, but continues for some time till the stationary population ($n_s$) is attained. This is usually about 2000. The most convenient measure of growth is the mean generation time (m.g.t.), *i.e.* the time required for the number of cells to double. Before the proliferation of the cells starts there is the usual lag phase, measured by extrapolating the logarithmic growth curve back to $n = n_0$. A typical curve is shown in Fig. 1. The lag depends upon the age of the inoculum: if the latter is very young, the lag is considerable, and has been called "early lag." As the cells of the inoculum age, the lag falls nearly to zero and then increases again. It is convenient to refer to cultures in the "early lag," "zero lag," or "late lag" state. The early lag is the time required to build up a critical concentration of a growth promoter which diffuses from the cells into the medium.[4, 5] In the late lag state various enzyme systems have lost their activity.

[1] Poole and Hinshelwood, *J. Chem. Soc.*, 1940, 1565.
[2] Tréfouel, J., Tréfouel, Mme. J., Nitti and Boret, *Compt. Rend. Soc. Biol.*, 1936, **120,** 756.
[3] Tréfouel, J., Tréfouel, Mme. J., Nitti, Boret and Fourneau, 1937, *ibid.*, **122,** 258.
[4] Lodge and Hinshelwood, *J. Chem. Soc.*, 1943, 213.
[5] Hinshelwood and Lodge, 1943, *in the press.*

**Form of the Growth Curve in Presence of Sulphonamides.**—This is shown in Fig. 1. When Bact. lactis aerogenes is grown for the first time in a medium containing the sulphonamide, the logarithmic phase is composite: the growth curve consists of two parts with an intersection at what we shall term the "transition point." Here growth with a longer m.g.t., which we shall call m.g.t. I, is superseded by growth with a shorter m.g.t. which will be called m.g.t. II.

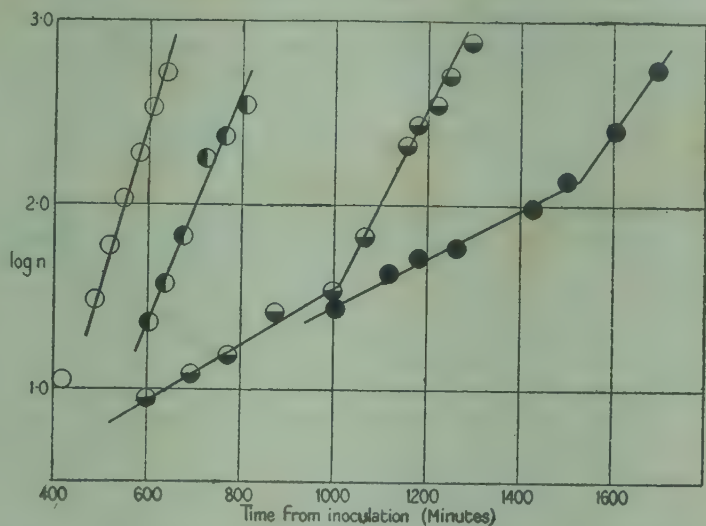


FIG. 1.—Typical Growth Curves. Open circles, normal culture: vertically divided circles, growth with 4·56 p.p. million sulphanilamide: horizontally divided circles—30·6 p.p.m. sulphanilamide (times plotted = actual times —600): full circles—467 p.p.m. sulphanilamide (actual times—1200).

**M.g.t. I and m.g.t. II as Functions of Sulphonamide Concentration.**—The retarding effect of the drug on logarithmic growth is best expressed by recording the ratio of the m.g.t. for normal growth to the observed value in presence of the sulphonamide. M.g.t. I and m.g.t. II both increase as the concentration increases (Fig. 2), *i.e.* the ratios, which measure the relative rates of growth, fall. They do not, however, fall to zero (as would, for example, be found with phenol), but to well-defined limits as seen in the figure.

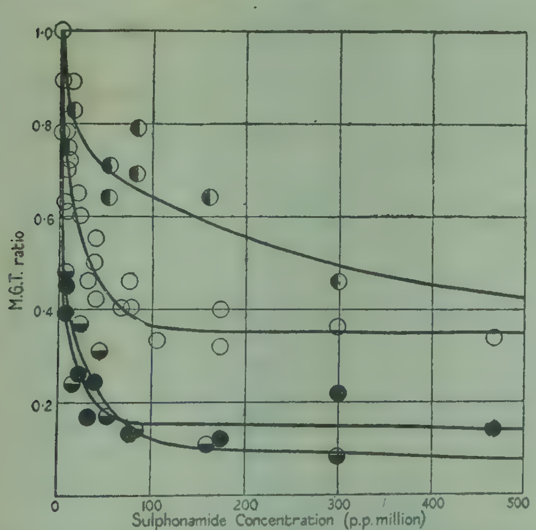


FIG. 2.—Influence of Drug Concentration on m.g.t. Ratio. Full circles—m.g.t. I in sulphanilamide (SA). Open circles—m.g.t. II in SA Horizontally divided circles—m.g.t. I in sulphaguanidine (SG). Vertically divided circles—m.g.t. II in SG.

**Early and Late Lag as Functions of the Sulphonamide Concentration.**—The lags all increase with the sulphonamide concentration: the results are given in Table I. The values for the early lags must be regarded as approximate only, since the extrapolation of the lower segment of the growth curve was uncertain with the small inocula used in early lag determinations, and an indirect method was used. The results, however, show that the lags increase steadily with increasing concentration, and show much less tendency to reach a limit than the mean generation times. Lag and m.g.t. are, in fact, separate and individual functions of the sulphonamide concentration.

**Interpretation of the Transition Point.**—This might have been interpreted as the point at which the cells have excreted enough of some

product which neutralises the action of the sulphonamide, or, in view of possible effects of *p*H on sulphonamide action,[6] as a point at which rapid change of *p*H occurs. Neither explanation fits. According to the first, the transition point would probably move to higher counts the greater the amount of drug to be dealt with. Actually it varies as in Fig. 4. The second is disproved by direct observation on the *p*H, which falls to about

TABLE I.—EFFECT OF SULPHONAMIDES ON LAG.

| Sulphanilamide Concentration (parts/million). | Late Lag (minutes). | Early Lag (minutes). | Sulphaguanidine Concentration (parts/million). | Late Lag (minutes). |
|---|---|---|---|---|
| *Series I.* | | | | |
| 0 | 925 | | 0 | 1575 |
| 2·2 | 971 | | 15·1 | 2034 |
| 4·4 | 1041 | | 29·1 | 2291 |
| 6·5 | 1102 | | 42·1 | 2678 |
| 20·2 | 1375 | | 82·5 | 3316 |
| 39·7 | 1474 | | 160 | 4958 |
| | | | 298 | Did not grow |
| *Series II.* | | | | |
| 0 | 863 | 340 | | |
| 20·2 | 2197 | 830 | | |
| 39·7 | 2736 | 660 | | |
| 107 | 2924 | 620 | | |
| 299 | 3555 | 2674 | | |
| 467 | 4798 | | | |

6·0 during the lag and, whether sulphonamide is present or not, remains nearly constant during most of the logarithmic phase.

The true explanation is suggested by the observation that subcultures taken toward the end of the second stage of the logarithmic phase show a much earlier transition point, and indeed may grow from the start with m.g.t. II. All the facts can be correlated by the following scheme. There are two modes of growth which may be referred to as I and II. I is the normal mode, and is sensitive to the presence of the drug. II is an alternative mode, not normally called into play, because it is less rapid than I. Ordinarily the enzymes involved in II are not immediately utilisable, but are in a state corresponding to the late lag phase. When mode I is largely put out of action by the sulphonamide, mode II, which is less sensitive to the drug, can compete on more equal terms. We have, therefore, a superposition of mode I with shorter lag and slower growth rate, and mode II with longer lag and more rapid growth. This is shown in Fig. 3, where the transition point is given by the intersection of the two growth curves. On subculturing cells which have once been grown in presence of the drug, the lag of mechanism II is shortened,

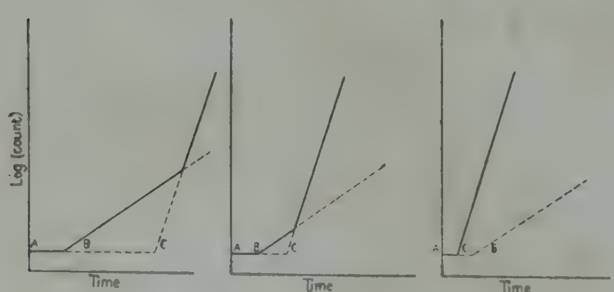


FIG. 3.—Adaptation by the Progressive Shortening of the Lag of a Reserve Mechanism. AB = lag of normal (but retarded) mechanism. AC = lag of reserve mechanism.

[6] Rose and Fox, *Proc. Soc. Exp. Biol. Med.*, 1942, **50**, 142.

with lowering of the transition point, as shown, or even complete replacement of mode I by mode II.

This view of the matter is confirmed by three sets of observations :—

(1) The lag in a medium containing sulphonamide is, in fact, less for cultures which have already been grown in presence of the drug (Table VII).

(2) The "immunity" of the subculture decays as the inoculum ages (Table VI). This is due to the normal development of lag with age. Ordinarily the lag of mode II is greater than that of mode I : when mode II has been mobilised by growth in sulphonamide its lag is shortened relatively to that of mode I, but on ageing of the cells there is reversion to the normal relationship.

(3) The curve of transition point against drug concentration is very different for sulphanilamide and for sulphaguanidine. This can be simply explained. Let the two modes of growth have lags $L_1$ and $L_2$ and growth constants $k_1$ and $k_2$ respectively, then

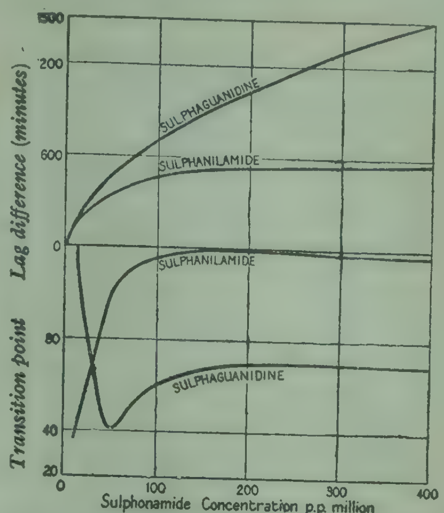


FIG. 4.—Lower Curves : Actual Variation of Transition Point with Drug Concentration. Upper Curves : Inferred Change of Lag Difference with Drug Concentration.

$$n_1 = n_0 e^{k_1(t - L_1)} \quad \text{and} \quad n_2 = n_0 e^{k_2(t - L_2)}$$

The transition point occurs where $n_1 = n_2 = n_t$, so that

$$L_2 - L_1 = 2{\cdot}303 \log \frac{n_t}{n_0}\left\{\frac{1}{k_1} - \frac{1}{k_2}\right\}.$$

From the dependence of $n_t$ on concentration in Fig. 4*b*, using the known dependence of $k_1$ and $k_2$ on concentration (Fig. 2), the variation of $L_2 - L_1$ has been calculated and plotted in Fig. 4*a*. The apparently quite different curves in Fig. 4*b* can be explained, as shown, by the relatively small quantitative differences in the effects of the two drugs on $(L_2 - L_1)$, which are not such as to cause surprise.

TABLE II.—EFFECT OF SULPHANILAMIDE ON STATIONARY POPULATION.

| Sulphanilamide Concentration (Parts/million). | $n_s$. |
|---|---|
| 0 | 920 |
| | 1440 |
| 2·2 | 1140 |
| 2·85 | 1300 |
| 4·4 | 1600 |
| 5·5 | 1760 |
| 6·5 | 1620 |
| 10·7 | 2340 |
| | 3240 |
| 20·2 | 3540 |
| | 2260 |
| 39·7 | 2760 |
| | 1600 |
| 54·6 | 2260 |
| | 1330 |
| 107 | 1600 |
| 299 | 730 |
| 467 | 780 |

**Stationary Population.**—The variation of $n_s$ with sulphonamide concentration is given in Table II. The final $p$H of sulphonamide cultures is higher than normal, which may in part explain the occurrence of a maximum.[7]

**Growth in Presence of Sulphonamides of Cultures in the "Zero Lag" State.**—This is anomalous, and can be explained by assuming that the sulphonamide requires time to exert its effect—either because it is slow in penetrating into the cell, or because it must suffer a preliminary chemical change.[8] Growth begins

[7] Lodge and Hinshelwood, *J. Chem. Soc.*, 1939, 1683.

[8] Main, Shinn, and Millon, *Proc. Soc. Exp. Biol. Med.*, 1938, **39**, 272, 591 ; 1939, **42**, 115 ; 1940, **43**, 593.

rapidly, at nearly the rate in the standard medium, but at an early stage it suffers an arrest, greater at high sulphonamide concentrations, after which the phenomena are as previously described. Fig. 5 shows some typical curves. As the lag of the inoculant lengthens, the arrest becomes more marked and finally the initial phase of rapid growth disappears.

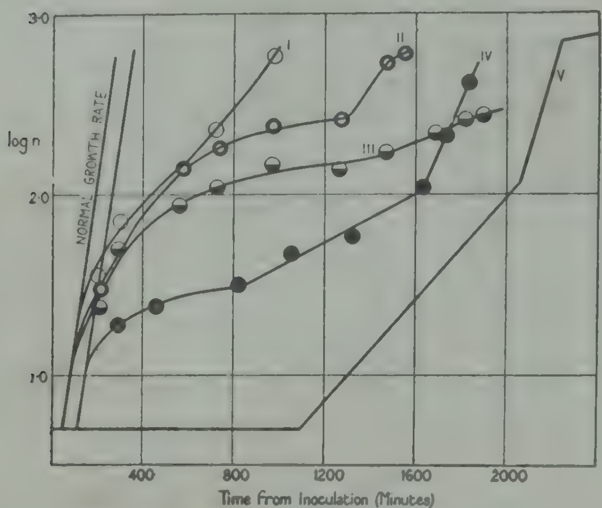


FIG. 5.—Initial Rapid Growth of Cultures with Short Lag. I. 107 p.p.m. SA, minimum lag. II. 299 p.p.m. SA, minimum lag. III. 467 p.p.m. SA, minimum lag. IV. 107 p.p.m. SA, lag 114 minutes. V. 299 p.p.m. SA, late lag.

**Influence of Aminobenzoic Acid and of Filtered Medium from Old Cultures.**—Aminobenzoic acid is known to antagonise the action of sulphonamides.[9] To determine whether it would produce a change similar to the transition from growth with m.g.t. I to that with m.g.t. II, the measurements in Table III were made. The aminobenzoic acid is found to restore the growth rate to normal, *i.e.* to reduce the m.g.t. I to well below m.g.t. II. What we have called mode II of growth, therefore, seems not to be associated with excess aminobenzoic acid production.

TABLE III.—EFFECT OF ADDITION OF FOREIGN SUBSTANCES TO SULPHANILAMIDE CULTURES.

Sulphanilamide concentration in parts/million = c.

(i) *Sulphanilamide alone.*

| | | | | | |
|---|---|---|---|---|---|
| $c$ = . | 0 | 10·7 | 39·7 | 107 | 299 |
| m.g.t. I. | 35′ | 88′ | 165′ | 220′ | 220′ |
| m.g.t. II. | — | 52·8′ | 68·9′ | 90·4′ | 97′ |
| lag (early) | 340 increasing to 1300 mins. | | | | |
| $n_s$ . . | 1300 | 3500 | 1500 | | |

(ii) *Sulphanilamide with 7·6 p.p.m. p-aminobenzoic acid.*

| | | | | | |
|---|---|---|---|---|---|
| $c$ = . | 0 | 10·7 | 39·7 | 107 | 299 |
| m.g.t. . | 36′ | 31·6′ | 32·6′ | 34′ | ca. 40′ |
| lag . | 330 | 231 | 213 | 113 | ca. 80 mins. |
| $n_s$ . . | 1486 | 1460 | 1600 | | |

(iii) *Sulphanilamide with 1/13 parts by vol. of centrifuged filtrate.*

| | | | | | |
|---|---|---|---|---|---|
| $c$ = . | 0 | 10·7 | 39·7 | | |
| m.g.t. I. | 36′ | — | 140′ | | |
| m.g.t. II. | — | 45·4′ | 79·2′ | | |
| lag . | 27′ | meaningless | | | |
| $n_s$ . . | 1140 | 2390 | 1700 | | |

Since the medium removed by filtration or centrifuging from a culture in the zero lag state has a very marked effect in reducing the early lag of

[9] Woods, *Brit. J. Exp. Path.*, 1940, **21**, 74.

inocula in the standard medium,[4] it was thought of interest to determine what effect this filtrate would have on sulphonamide action. The usual reduction of lag is observed, but no neutralisation of the other actions of the drug (Table III).

**Growth in an Asparagine Medium.**—When the ammonium sulphate in the standard medium is replaced by asparagine, the effects of the sulphonamide remain qualitatively the same, but the relative values of the m.g.t.'s are changed. M.g.t. I in the asparagine medium is uniformly higher, *i.e.* the growth mechanism which utilises the amino acid is more sensitive to the action of the drug. On the other hand, m.g.t. II, that of the reserve mechanism, is the same for both media (Table IV). The adaptation

TABLE IV.—Growth in Presence of Sulphanilamide with Alternative Nitrogen Sources.

| Nitrogen Source. | Sulphanilamide Concentration (parts/million). | Lag (mins.). | m.g.t. I. (mins.). | m.g.t. II. (mins.). |
|---|---|---|---|---|
| Ammonium Sulphate | 117 | 2000 | 194 | 76 |
| | 225 | 4075 | 220 | 57 |
| Asparagine | 117 | 2275 | 386 | 90 |
| | 225 | 1500 | 1545 | 60 |

phenomena described below occur in the same way whichever nitrogen source is used, either for training or for testing.

**Adaptation of Bact. lactis aerogenes to Growth in Presence of Sulphonamides: Training Phenomena.**—The immunity towards sulphonamides developed by the cells after growth in their presence has already been referred to. The development of this immunity has been studied in some detail. For the quantitative measure of it we define a "training coefficient" as follows: let $t$ be the time required for the count of the culture to increase from 20 to 200, then

$$T = \frac{t_{\text{trained}} - t_{\text{untrained}}}{t_{\text{untrained}} - {}_{\text{trained}}}.$$

The progress of training may, according to circumstances, be reflected in a shift of the transition point to lower values of $n$, or in a decrease in the value of m.g.t. II. Both increase $T$. An untrained culture has $T = 0$, one for which the transition point has been so far lowered that all growth occurs with the m.g.t. II of the first growth in sulphanilamide has $T = 0{\cdot}8$, while one so completely trained that it grows like a normal culture in absence of drug has $T = 1{\cdot}0$.

**Complete Adaptation.**—After 23 or 30 subcultures in a medium containing 216 p.p. million sulphanilamide the characteristics of the final cultures were as follows: (1) $T$ approached 1·0. (2) $T$ did not decrease much as the culture aged. (3) The adaptation was non-specific: cultures trained in sulphanilamide showed a high degree of immunity to sulphaguanidine. (4) $T$ did not show a rapid or serious decline after several passages through the standard medium containing no sulphonamide and subsequent test in presence of sulphanilamide. These facts are illustrated in Table V.

**Development of Adaptation. First and Second Cultures in Sulphonamide Media.**—The chief facts are shown in Figs. 6 and 7. After one passage through sulphanilamide the cells are well enough trained to grow in a second culture at m.g.t. II throughout. This training is specific: when identical inocula from a sulphanilamide culture are transferred to media containing sulphanilamide and sulphaguanidine respectively, they

are trained towards the former but not towards the latter, and *vice versa* (Fig. 6). The training is shown both in the reduction of lag, and in the earlier transition point.

TABLE V.—GROWTH OF CELLS SUBCULTURED 30 TIMES IN PRESENCE OF SULPHANILAMIDE.

(i) *Growth after passage through normal medium.*

| No. of Passages (Total Time in Brackets). | m.g.t. II. (mins.). | Training Coefficient T in SA 216 p.p.m. |
|---|---|---|
| 0 (completely trained strain) | 27·6 | 1·03 |
| 1 (29 hours) | 44·4 | 0·73 |
| 2 (100 hours) | 43 | 0·81 |
| 5 (15 days) | 46 | 0·88 |
| 5 times in artificial medium as above, then 3½ weeks in bouillon | 45·6 | 0·81 |
| After 3½ weeks in bouillon and then 16 sub-cultures in normal medium | 43·6 | 0·89 |
| Ditto and 17 sub-cultures in normal medium | 41·4 | 0·76 |

(ii) *Variation of adaptation with age of inoculant.*

| Time from Onset of Stationary Phase of Parent (mins.). | T in 225 p.p.m. Sulphanilamide. | T in 160 p.p.m. Sulphaguanidine. |
|---|---|---|
| 0 | 1·03 | 0·99 |
| 1090 | 0·87 | 0·85 |
| 2555 | 0·67 | 0·80 |
| 4040 | | 0·84 |

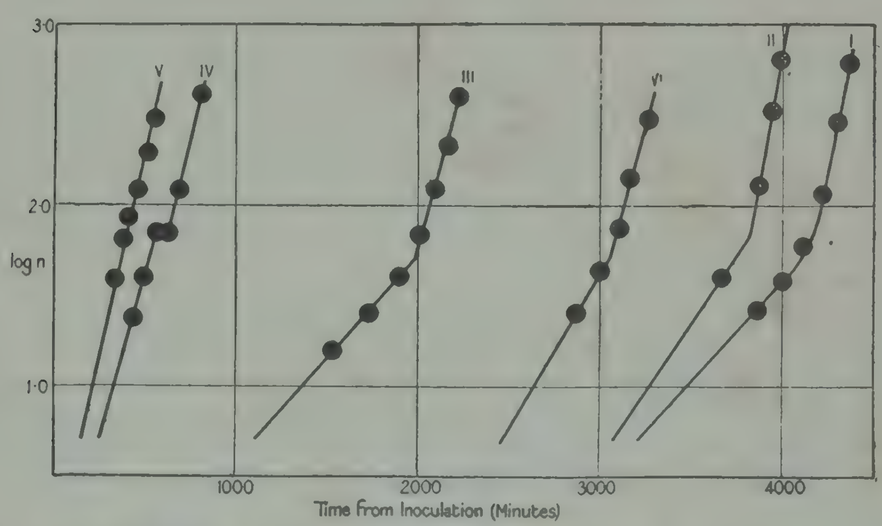


FIG. 6.—Early Stages of Adaptation to Sulphonamides. I. Untrained growth in SG (160 p.p.m.). II. Untrained growth in SA (218 p.p.m.). III. Culture in 160 p.p.m. SG. IV. Culture in 225 p.p.m. SA; III. and IV. both inoculated at same time from a parent culture in 225 p.p.m. SA. V. Culture in 160 p.p.m. SG. VI. Culture in 225 p.p.m. SA; V. and VI. both inoculated at same time from parent culture in 160 p.p.m. SG.

TABLE VI.—CHARACTERISTICS OF ADAPTATION AT SECOND SERIAL SUBCULTURE IN PRESENCE OF SULPHONAMIDE.

SA = sulphanilamide. SG = sulphaguanidine.

(a) *Effect of variation in age of parent.* Parent and test cultures contain 220 p.p.m. SA.

| Age, measured from Onset of Stationary Phase. (mins.). | m.g.t. II (mins.). | *T*. |
|---|---|---|
| *Series I.* | | |
| −460 | 67 | 0·13 |
| 0 | 74·6 | 0·58 |
| 807 | 82·0 | 0·35 |
| *Series II.* | | |
| 0 | 88 | 0·25 |
| 2410 | 58·4 | 0·13 |
| 3890 | 96 | 0·02 |
| 4650 | 52 | −0·04 |
| 6355 | — | 0·01 |

(b) *Change of immunity to SA and SG with age of parent.* Parent grown in 225 p.p.m. SA.

| Age, measured from Onset of Stationary Phase. (mins.). | *T* in 225 p.p.m. SA. | *T* in 160 p.p.m. SG. |
|---|---|---|
| 0 | 0·69 | 0·26 |
| 928 | 0·32 | 0·28 |
| 2246 | 0·2 | −0·18 |

(c) *Loss of immunity after passage through normal medium.*

| | Lag (mins.). | m.g.t. I (mins.). | m.g.t. II (mins.). | *T*. |
|---|---|---|---|---|
| Parent (3rd culture in 220 p.p.m. SA) . . . . . . | 30 | — | 62 | 0·70 |
| Culture in 220 p.p.m. SA after growth in normal medium . . | 280 | 198 | 58·2 | −0·14 |

(d) *Effect of growth in one drug on immunity toward another.*

| Drug in Inoculent Culture. | Drug in Test Culture. | Lag (mins.). | m.g.t. I (mins.). | m.g.t. II (mins.). | *T*. |
|---|---|---|---|---|---|
| 220 p.p.m SA . | 220 p.p.m. SA | 250 | — | 78·0 | 0·33 |
| 220 p.p.m. SA . | 160 p.p.m. SG | 1060 | 264·0 | 80·8 | −0·12 |
| 160 p.p.m. SG . | 220 p.p.m. SA | 2430 | 194·0 | 76·0 | 0·12 |
| 160 p.p.m. SG . | 160 p.p.m. SG | 120 | — | 68·0 | 0·65 |

After one passage through the standard medium without sulphonamide the immunity is largely lost (Fig. 7). The immunity has not been attained until some time past the transition point, as shown by one of the curves in Fig. 7.

The immunity decays as the culture ages (Table VI).

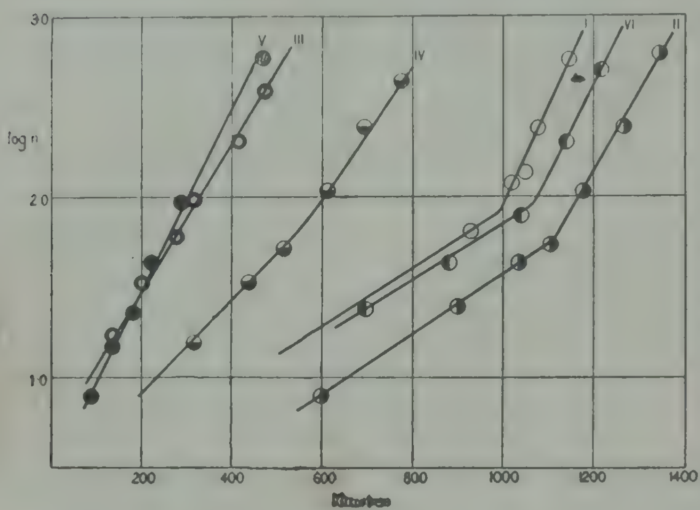


FIG. 7.—Development and Decay of Adaptation in Early Stages. All cultures in 218 p.p.m. SA. I. First culture (time zero 2000 minutes from origin). II. Second culture in SA inoculated at transition point. III. Second subculture, inoculated at onset of stationary phase. IV. Second subculture, inoculated 800 minutes after onset of stationary phase. V. Strain made immune by three passages through SA, inoculated at onset of stationary phase. VI. Strain, from V as parent, whose adaptation has been lost after passage through normal medium.

The lag is in any case a function of the age of the inoculum, but for inocula of comparable ages the lag is much less in the second sulphanilamide culture than the first (Table VII).

**Progressive Development of the Adaptation.**—Repeated subcultures were made in the standard medium containing 225 p.p. million sulphanilamide. At frequent intervals the following quantities were determined: (1) $T$ in sulphanilamide, (2) $T$ in sulphaguanidine, (3) $T$ in sulphanilamide of the subculture which had been passed in the meantime through the standard medium containing no sulphonamide, (4) the value of m.g.t. II in the sulphanilamide medium. The results are shown in Fig. 8, which reveals the following facts. The specific immunity to one drug develops more rapidly than the non-specific immunity to both, though the final state is complete adaptation both to sulphanilamide and to sulphaguanidine. The irreversibility of the adaptation also develops according to its own characteristic curve.

TABLE VII.—VARIATION OF LATE LAG WITH AGE OF PARENT IN SULPHANILAMIDE CULTURES.

The zero of the age scale is taken as the point at which the count of the parent is 1000.

All test cultures contained 225 p.p.m. of sulphanilamide.

| Count of Inoculant. | Age (mins.). | Lag (mins.). |
|---|---|---|
| (a) *Normal culture as parent.* | | |
| 1400 | 310 | 990 |
| 1440 | 958 | 2410 |
| — | 1645 | 2140 |
| 1900 | 3020 | 3940 |
| (b) *Culture grown in 225 p.p.m. sulphanilamide as parent.* | | |
| 1000 | 0 | 14 |
| 1300 | 910 | 265 |
| 1340 | 1508 | 1421 |
| 2080 | 2810 | 2060 |

For the earlier serial subcultures the increase in the value of $T$ is due principally to the lowering of the transition point, m.g.t. II showing little change. In the later stages of the training process, m.g.t. II itself begins to decrease and ultimately falls nearly to the standard value for normal growth.

Non-specific immunity of bacteria to sulphonamides after repeated subculture in their presence has been reported by Kirby and Rantz.[10] Table VIII shows that adaptation can occur quite readily in presence of

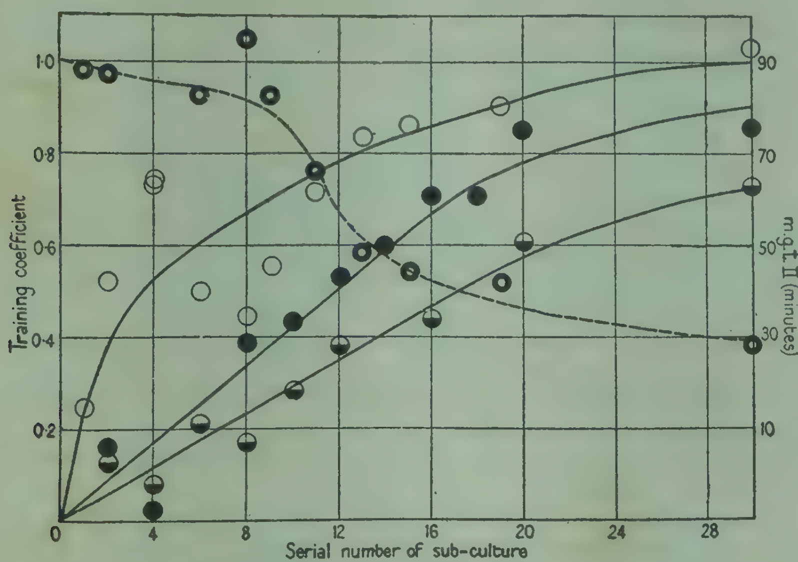


FIG. 8.—Progressive Development of Training in Presence of SA. Open circles, training coefficient, *T*, in SA. Full circles, *T* for SG. Horizontally divided circles, *T* in SA after passage through normal medium. Black rimmed circles, m.g.t. II in SA (ordinates on right of figure).

TABLE VIII.—GROWTH AND ADAPTATION IN MEDIA CONTAINING TWO SULPHONAMIDES.

SA = sulphanilamide. SG = sulphaguanidine.

| Drug in Parent Culture. | Drug in Test Culture. | Lag (minutes). | m.g.t. I (minutes). | m.g.t. II (minutes). | *T*. |
|---|---|---|---|---|---|
| None | 113 p.p.m. SA, 80 p.p.m. SG | 2800 | 211·2 | 90·4 | 0 |
| None | 160 p.p.m. SG | 2200 | unknown | 45·2 | 0 |
| 225 p.p.m. SA | 113 p.p.m. SA, 80 p.p.m. SG | 1700 | 235·2 | 71·2 | 0·2 |
| 225 p.p.m. SA | 160 p.p.m. SG | 1850 | 194·8 | 65·2 | −0·48 |
| 113 p.p.m. SA, 80 p.p.m. SG | 225 p.p.m. SA | 35 | — | 53·0 | 0·6 |
| | 113 p.p.m. SA, 80 p.p.m. SG | 25 | — | 64·6 | 0·67 inoc. from same culture at same time. |
| | 160 p.p.m. SG | 40 | — | 49·4 | 0·54 |

a mixture of sulphanilamide and sulphaguanidine, *i.e.* there is nothing in the mechanism of one specific type of training which excludes the simultaneous occurrence of the other.

[10] Kirby and Rantz, *J. Exp. Med.*, 1943, **77**, 29.

## Discussion.

### (*a*) Formal Interpretation of Results.

(1) Since the final adaptation to the sulphonamide is complete, non-specific and difficultly reversible, and since various substances such as aminobenzoic acid or methionine [11] are known to antagonise the action of the drugs, the simplest hypothesis to make is that during training the cells develop the enzymes necessary for the synthesis of an appropriate sulphonamide antagonist. Once developed, these enzyme systems, for reasons which will be discussed below, are relatively permanent.

(2) In the earlier stages the adaptation is different: it is specific, easily reversible, not only by subculture in the normal medium, but by simple ageing of the cells, and manifests itself chiefly by a lowering of the transition point. As we have seen, it is interpretable as a shortening of the lag phase of a reserve growth mechanism. The mobilisation of this mechanism is, however, but a temporary expedient, which serves while the more radical solution is being developed.

(3) The initial adaptation being little more than the shortening of a lag, it is easy to understand why in the early stages of training the immunity is readily lost: the natural lengthening of lag, as cultures age, is a phenomenon of general occurrence.

(4) The complex effects of the sulphonamides upon growth suggest that the drugs do not so much interfere with a single link in the series of vital processes as bring about a general disorganisation of the cell. The response of the cell is correspondingly complex.

(5) The curves in Fig. 2 show that growth cannot be entirely inhibited. If we think of the enzymes responsible for the cell processes as constituted by protein patterns of various kinds, then we must conclude that there are certain groupings which are not affected by the drugs and which are capable of serving the needs of the cell, though less efficiently than those in normal use.

(6) The patterns which serve as the basis of enzyme functions may be almost infinitely varied. In the presence of sulphonamide, fresh configurations may be slowly built up which can perform the essential reactions of the cell in spite of the adverse conditions. This may well occur during the lag phase of the mechanism corresponding to m.g.t. II. Since the immunity which develops is specific to one drug, quite delicate structural or stereochemical relations seem to be involved at this stage.

It is important to note that the specificity is reciprocal in the early stages of immunisation, *i.e.* training in sulphaguanidine does not immunise to sulphanilamide, nor *vice versa*. (It might have been found that the drugs could be arranged in an order of potency, training in the presence of the more powerful members of a series immunising towards all the less powerful members.)

### (*b*) Natural Selection and Adaptation.

Where repeated sub-culture changes the character of a bacterial population, two hypotheses are possible. First, we may assume the initial population to have been made up of several distinct strains, one of which increases relatively to the other during successive growth cycles. This we shall call selection, restricting the term to a mere shift in the numerical balance between non-interconvertible types. Secondly, we may suppose that in successive generations the cells of one initial type undergo modification of their enzyme systems. This we shall call adaptation.

In many respects the consequences of the two hypotheses are the same, but in interpreting the above results we prefer the latter for the following reasons: (1) In the early stages of training the adaptation is lost on

[11] Bliss and Long, *Bull. Johns Hop. Hosp.*, 1941, **69**, 14.

passage through the normal medium. This reversal, according to the selection hypothesis, would mean that the postulated sulphonamide-resistant strain actually grew less well in the normal medium : an assumption which is not impossible, but unlikely. (2) Immunity decreases with the age of the culture. This would mean that the type which grew most readily in presence of sulphonamide also died off most rapidly : again, a very unlikely conclusion. (3) The training is a multiple process, different functions adapting themselves at different rates, as shown by Fig. 8. In terms of the selection theory this would mean that there were present initially, not two but a whole spectrum of bacterial strains. Since the culture used was originally derived from a single colony, this could only be true if one original type had at one stage become heterogeneous—an assumption which itself admits the possibility of continuous modification of the strain. It is therefore simpler and more logical to explain the observed changes by assuming an actual adaptation of the enzyme systems.

### (*c*) Nature of the Adaptation Process.

(1) We regard adaptation as a modification of the enzyme balance of the cell.

(2) In particular, when a certain function of the cell develops during the adaptation process, we imagine that there occurs an expansion of certain protein patterns.

(3) We now ask why this expansion occurs in response to a need of the cell. We believe the answer to be that it will, in fact, occur automatically when some key process can be formulated according to the general scheme :

$$\textit{enzyme pattern} + \textit{reactant molecule} = \begin{matrix}\textit{extended enzyme}\\ \textit{pattern}\end{matrix} + \textit{molecular fragments}$$

(*if necessary utilisable in further reactions*),

the enzyme expanding as it does its work. Its amount will then increase with time according to the law $x = x_0e^{kt}$ during certain periods.

(4) An example of adaptation reduced to its simplest terms may now be considered. Let there be two enzyme systems present in the cell, the first in very small amount $x_0$, and the second in large amount $y_0$. Let both operate according to the above scheme. Consider growth in a medium in which $x_0$ increases at a small rate $k_x$ while $y_0$ increases at a greater rate $k_y$. The cell divides when it reaches a certain size, at which time $y_0$ will have expanded to some amount $y_1$ while $x_0$ will only have increased to $x_1$, where

$$\frac{x_1}{y_1} = \frac{x_0}{y_0}e^{(k_x - k_y)t}.$$

Now let the cell be transferred to a medium where the essential reactants for the increase of "y" are in short supply, so that the specific rate $k_y$ is reduced to $k'_y$. The cell now makes more use of the products of the $x$-enzyme. Let division still occur when $y_1$ is reached : the value of $x_1$ at this point is now given by $\frac{x'_1}{y_1} = \frac{x_0}{y_0}e^{(k_x - k'_y)t}$, so that $\frac{x'_1}{x_1} = e^{(k_y - k'_y)t}$. Thus the new cell starts with a better supply of enzyme "x" than did its parent. When it, in turn, divides, we have $\frac{x''_1}{x_1} = e^{(k_y - k'_y) \cdot 2t}$, and so on, until enzyme "x" takes control and the moment of division depends upon the attainment of a given value of $x_1$. If the two enzymes are independent, and concerned in alternative, rather than in linked mechanisms, their amounts cannot stand in a fixed relation during cell growth, unless the two rate constants are identical, which would not in general be true.

Naturally in real examples the adaptation would be a complicated combination of schemes of which the above is a crude illustration only.

(5) The fundamental scheme in paragraph (3) of this section has simple analogies in other parts of chemistry. The decomposition of arsine is catalysed by a surface of metallic arsenic, so that we have :

*catalyst + substrate = more catalyst + product.*

This scheme can be extended to any case where an ordered structure expands by the accretion of new units. The order thereby created represents a decrease in free energy which can compensate accompanying increases, in the formation of other reactive products. Certain protein patterns may well extend themselves by removing suitable fragments from other molecules, leaving residues which can participate in other cell reactions. If this type of coupling lies at the basis of the equation in paragraph (3), then adaptation can be understood in principle.

(6) From the above, it seems that an enzyme system will remain in use only when it is the most efficient means available for a particular purpose. To explain the persistance, during growth in the normal medium, of the antagonist-forming mechanism invoked to explain adaptation to the sulphonamide (see above), we must assume that this process can also be used with advantage in normal growth. Such a circumstance is by no means unlikely.

## Summary.

The influence of two typical sulphonamide compounds (sulphanilamide and sulphaguanidine) on the growth of Bact. lactis aerogenes has been studied. Increasing concentrations of either drug progressively lengthen the lag phase and reduce the growth rate. The latter, however, never reaches zero, as it does in presence of other antiseptics.

At a certain stage during growth in presence of the sulphonamide there may be a transition from a slower to a more rapid rate of multiplication. This has been shown to be due to an adaptation of the bacteria.

After thirty passages through media containing sulphonamide an immunity has developed which is almost complete : it is neither specific to a particular sulphonamide, nor reversible by passage through the normal medium. The growth rate may reach that found in sulphonamide free media. To explain this it is assumed that the cells develop enzymes which produce a sulphonamide antagonist. The establishment of this complete immunity is slow and complex. In the early stages of training the adaptation is specific to one drug, easily lost, and partial, in that the growth rate is markedly less than normal. The immunity attained after one or two passages through the sulphonamide medium is of a different kind from that developed later, and can be interpreted as the shortening of the lag of an alternative growth mechanism which is more resistant to sulphonamides than that normally concerned.

The physico-chemical basis of the adaptive process is considered.

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## *GENERAL DISCUSSION*

**Prof. Rideal** said : Has Mr. Lodge had any evidence of the treated bacteria which showed abnormal type of growth reverting to the normal pattern when put back into normal media for several generations. I remember that during the last war I carried out a few experiments in the Somme valley on the fermentation of sugars by B.-coli and found that I could modify the fermentative properties of B.-coli for various sugars by treatment with hypochlorite. In this case the recovery of fermentative powers on sub-incubation was not achieved.

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* Page numbers in Clarendon type refer to papers contributed for Discussion.



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